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Functional analysis of the CAF1 protein

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FUNCTIONAL ANALYSIS OF THE CAF1 PROTEIN

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DEDICATION

Dedicated to my wife, Jeongsook Yoon

And

To my parents

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ABSTRACT

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The CAF1 protein is a component of the CCR4-NOT deadenylase complex. While yeast CAF1 displays deadenylase activity, this activity is not required for its function in vivo, and CCR4 is the primary deadenylase in the complex. In order to identify CAF1-specific functional regions required for deadenylaton in vivo, we targeted for mutagenesis six regions of CAF1 that are specifically conserved among CAF1 orthologs. Defects in three regions of the CAF1 protein (residues 173-175, residues 255-257 and residues 340-342, alleles *caf1-1*, *caf1-3* and *caf1-6*, respectively) were found to dramatically reduce the rate of deadenylation in vivo and to result in typical *caf1* deletion phenotypes without critically affecting the ability of CAF1 to bind CCR4. In contrast, defects in residues 213-215, which were defined as the site required for binding CCR4 (*caf1-2* allele), reduced the rate of deadenylation to a lesser extent than did *caf1-1*, *-3* or –*6* and its in vivo phenotypes were correspondingly less severe than these other alleles. The *caf1-1*, *-3*, and *-6* alleles or a *caf1* deletion, unlike that of *ccr4* or *caf1-2*, were synthetically lethal with defects in DHH1, a decapping regulator that is involved in controlling translation, and with certain defects in poly(A) binding protein (PAB1) that display decreased rates of translation and deadenylation. A *caf1* deletion also had a more

significant effect on translation than did *ccr4*. These and other genetic experiments suggest a role for CAF1 in translation separate from that of CCR4. Conversely, a *pab1* translation defect that did not affect deadenylation by itself or with *ccr4* severely blocked deadenylation when coupled with a *caf1* deletion. These results support a role for CAF1 in communications between translation and deadenylation that are required for both processes.

GENERAL INTRODUCTION

The regulation of gene expression is a fundamental aspect of all biological processes. The degradation of eukaryotic mRNA plays significant roles in modulating gene expression since the mRNA pool in the cell is determined both by the rates of synthesis and of degradation. In eukaryotes, the regulated decay of mRNAs can occur through two general mechanisms: deadenylation-dependent and -independent decay pathways (Figure 1). Monitoring of mRNA fidelity can be performed through specialized decay mechanisms such as nonsense-mediated decay (NMD) and nonstop decay (Figure 1).

1. Deadenylation-dependent mRNA decay pathways

1.1. Deadenylation

A principal mRNA degradation pathway used in eukaryotes is usually initiated by removal of the 3' poly(A) tail. Evidence that the poly(A) tail removal is required for mRNA degradation comes from assessing the kinetics of deadenylation relative to the decay of several yeast and mammalian mRNAs (Brewer and Ross 1998; Shyu et al., 1991; Decker and Parker, 1993). Transcriptional pulse-chase experiments showed that these mRNAs do not degrade completely until their poly(A) tails have been shortened. Moreover, mRNAs that are known to be unstable are also deadenylated more rapidly than a stable mRNA. The unstable *MFA2* transcript, for instance, was deadenylated with a 3-

fold higher rate (~13 A's/min) than the stable *PGK1* transcript (~4 A's/min) (Decker and Parker, 1993). Thus, deadenylation is an initial rate limiting step in mRNA decay.

The understanding of the mechanisms of mRNA degradation is mainly based on studies in *Saccharomyces cerevisiae*. Deadenylation of the 3' poly(A) tail to a length that is too short to bind the major poly(A)-binding protein, PAB1, leads to one of two different degradation pathways in yeast (Figure 2). The loss of PAB1 leads to disruption of the eIF4F translation initiation complex (consisting of eIF4E, eIF4G and PAB1) which is bound to the 5' cap structure via the cap binding protein eIF4E. The destabilized mRNP structure causes decapping followed by final 5'-3' exonucleolytic cleavage by XRN1 (Figure 2) (Hsu and Stevens, 1993).

1.2. Decapping

Decapping (5' cap hydrolysis) depends on almost complete deadenylation (5~10A's) and has been proved to be a highly conserved decay mechanism in eukaryotic cells (Dunckley and Parker, 1999; Lykke-Andersen J., 2002; Wang et al; 2002). First, the decapping enzymes, DCP1 and DCP2, are conserved among eukaryotes (Dunckley and parker, 1999; Lykke-Andersen J. 2002; Wang et al., 2002). Secondly, decapped mRNAs are specifically detected on mammalian transcripts with short poly(A) tails (Couttet et al., 1997). More recently, decapping components were found to be aggregated in well-conserved discrete cytoplasmic foci termed P-body (Sheth and Parker, 2000; van Dijk et al., 2002; Lykke-Andersen, 2002). This specialized cellular structure is also known to be involved in translational control that is very similar to another form of mRNA containing cytoplasmic particles, referred to as a stress granule (Kedersha and Anderson, 2002).

Decapping is catalyzed by the decapping complex which is composed of DCP1 and DCP2. DCP1 is absolutely required for *in vivo* decapping process but the recombinant protein is inactive. Thus, DCP1 has been suggested to undergo a DCP2-dependent activation to become decapping competent (Dunckley et al., 2001). DCP2 is a member of the Nudix family of pyrophosphatases, emzymes that hydrolyse nucleoside diphosphates and mutations in the DCP2 Nudix motif inactivate decapping activity *in vivo* and *in vitro* (Dunckley and Parker, 1999; Lykke-Andersen J., 2002; Steiger et al., 2003; Wang et al., 2002). The recombinant DCP2 protein is active *in vitro* and releases m^7GDP from a capped RNA, leaving a 5' monophosphate on the RNA. It also showed a capped RNA substrate preference as the rate of hydrolysis of free m^7GpppN is very low. The activity increases significantly when the length of the RNA is increased from around 20-50 to ~100 nucleotides (Steiger et al., 2003; Stevens, 1998; van Dijk et al., 2002), suggesting the existence of an RNA-binding site on DCP2 that is distinct from the active site. These RNA binding sites were subsequently identified in the C-terminal part of the Nudix domain (Piccirillo et al., 2003).

Several proteins have been identified as the regulators of the decapping process. EDC1 and EDC2 proteins stimulate decapping in yeast and these two proteins have sequence similarity to each other. EDC1 associates with the DCP1/DCP2 complex (Dunckley et al., 2001). Mutations in the *PAT1* gene reduce the rate of decapping *in vivo* but have no effect on cap hydrolysis *in vitro* suggesting that it is not directly involved in the catalytic step (Bonnerot et al., 2000; Bouveret et al., 2000; Hatfield et al., 1996). PAT1 is associated with the LSM complex which also promotes decapping probably by binding the 3' end of the mRNA (He and Parker, 2001). Another protein associated with the LSM complex is DHH1, a member of the DEAD box ATPase family. The homolog of

DHH1 in higher eukaryotes is the rck/p54 protein, which has been demonstrated to have ATP- or GTP-dependent RNA helicase function (Cougot et al., 2004; Ladomery et al., 1997; Minshall et al., 2001). Of these decapping stimulators, PAT1 and DHH1 have also reported to be involved in translation (Wyers et al., 2000; Coller and Parker, 2005).

1.3. 5' to 3' decay

5'-3' exonucleolytic cleavage is the final step of the major deadenylation dependent mRNA decay pathway. This step is conducted by XRN1, a divalent cation-dependent processive 5'-exonuclease. Capped RNA is resistant to XRN1 cleavage, and RNA carrying a 5' monophosphate is strongly preferred over 5' hydroxylated RNA. This enzyme is also evolutionarily conserved among eukaryotes (Bashkirov et al., 1997; Till et al., 1998; Newbury and Woollard, 2004).

1.4. 3' to 5' decay

Eukaryotic mRNAs can also be degraded in a 3' to 5' direction following deadenylation by the exosome, a complex of 3'-5' exonucleases (Muhlrad et al., 1995; Jacobs Anderson and Parker, 1998). It is clear that the 3' to 5' degradation pathway is considerably slower in yeast than decapping and subsequent 5' to 3' degradation (Muhlrad et al., 1995; Anderson and Parker, 1998). However, either pathway by itself is sufficient to support cell viability (Anderson and Parker, 1998). Mutations that inhibit both pathways are synthetically lethal, which suggests that these are the only two general pathways of mRNA decay in yeast. The exosome exists both in the nucleus and in the cytoplasm. The nuclear and the cytoplasmic exosome from yeast share a set of ten subunits. Nine of these form a stable core complex except RRP44 which is loosely

associated (Allmang et al., 1999; Mitchell and Tollervey, 2000). Six subunits, RRP41/SKI6, RRP42, RRP43, RRP45, RRP46, and MTR3, have sequence similarity to *E. coli* polynucleotide phosphorylase (PNPase) and RNase PH, suggesting that like these two enzymes they catalyze the 3'-5' degradation of RNA by phosphorolytic attack, releasing nucleoside 5' diphosphates (Allmang et al., 1999; Mitchell et al., 1997). Two additional subunits of the yeast exosome, RRP4 and RRP44, have hydrolytic 3' exonuclease activities (Mitchell et al., 1997). The RRP40 subunit may also be a hydrolytic enzyme, based on its sequence similarity to RRP4. No catalytic activity has been predicted for the CSL4/SKI4 subunit. Four additional polypeptides are not stably associated with the exosome but contribute to exosomal mRNA degradation in the cytoplasm: SKI2, SKI3, SKI7, and SKI8. These proteins are not involved in nuclear functions of the exosome (Jacobs Anderson and Parker, 1998; van Hoof et al., 2000). SKI2, SKI3, and SKI8 form a complex (Brown et al., 2000) and SKI2 is a predicted RNA helicase.

2. Deadenylation-independent mRNA decay pathways

2.1. Nonsense-mediated decay (NMD)

mRNA decay can also be initiated by decapping and 5'-3' decay of the transcript independent of poly(A) shortening. Eukaryotic cells has evolved complex mRNA surveillance mechanisms to increase the fidelity of gene expression by monitoring and degrading aberrant mRNAs that, if translated would produce truncated proteins that is often harmful to the cell viability. For instance, aberrant mRNAs containing a premature translational stop codon are decapped without prior poly(A) shortening (Cao and Parker,

2003; Muhlrad and Parker, 1994). This rapid degradation of aberrant transcripts is referred to as nonsense-mediated decay (NMD). In yeast, NMD involves the normal translational machinery which, upon recognition of a premature termination codon, triggers decapping without prior deadenylation thereby allowing the 5' to 3' exonucleolytic cleavage by XRN1 (Muhlrad and Parker, 1994; Hagan et al., 1995; Beelman et al., 1996; Hatfield et al., 1996). Additional proteins that are required in NMD are the UPF1, UPF2 and UPF3 proteins. UPF1 has been purified from yeast and shown to contain RNA binding as well as RNA-dependent ATPase and RNA helicase activities (Czaplinski et al., 1995; Weng et al., 1996). UPF2 is an acidic protein without significant homology to other proteins and UPF3 is a protein that is capable of shuttling between the nucleus and cytoplasm (He and Jacobson, 1995). These proteins cause rapid decapping without deadenylation by recognizing an improper translation termination and interacting with the translation termination factor eRF3 (Weng et al., 1996).

2.2. Non-stop decay (NSD)

Transcripts lacking termination codons are also subjected to rapid degradation; however, the mechanism is quite different from NMD. Nonstop decay (NSD), unlike NMD, is localized to the cytoplasmic compartment in both mammalian and yeast cells. This process is performed by the exosome nuclease complex and begins at the 3'-poly(A) tail. This exosomal nuclease activity requires SKI7 which is an exosome-associated protein and related to the translation elongation factor EF1A and the translation termination factor eRF3. Therefore, the likely function of SKI7 is to distinguish nonstop from normal mRNAs by binding to the empty A site of stalled ribosomes that have reached the 3' end of a mRNA. This interaction could bring the

exosome to the mRNA as SKI7 interacts with both the ribosome and the exosome (van Hoof et al., 2002; Frischmeyer et al., 2002).

2.3. Endonucleolytic Cleavage

Eukaryotic mRNAs can be degraded via endonucleolytic cleavage prior to deadenylation. Evidence for this mechanism comes from the analysis of transcripts, such as *c-myc*, insulin-like growth factor II (*IGF-II*), mammalian *9E3*, serum albumin, transferring receptor, vitellogenin mRNA, and Xenopus *β-globin* mRNA where fragments are detected *in vivo* that correspond to the 5' and 3' portion of the transcript and are consistent with internal cleavage with the mRNA (Binder et al., 1994; Bremer et al., 2003; Hanson and Schoenberg, 2001; Stoeckle and Hanafusa, 1989; van Dijk et al., 2001). It is likely that a variety of endonucleases may exist with different sequence-specific cleavages because there does not appear to be any similarity between the cleavage sites in these mRNAs. For example, the endonuclease which is responsible for cleaving *β-globin* mRNA appears to be recognize at UG and UC dinucleotides (Bremer et al., 2003), whereas the decay of *eNOS* pre-mRNA appears to occur by cleavage at CA repeats (Hui et al., 2003). Recently, endonucleolytic cleavage mechanism has found to be the major pathway of RNA induced silencing process (RNAi). RNAi response is initiated by the recognition of dsRNA by the ATP-dependent endonuclease Dicer that cleaves dsRNA to generate 21-23 nucleotide RNA species used to target the RNAi-induced silencing complex (RISC) to the complementary mRNA.

3. Control of mRNA deadenylation

3.1. *cis*-acting elements

The control of mRNA degradation is often coded inside the mRNA body which is usually found in the 3' untranslated region (3'-UTR). Several *cis*-acting elements stimulating the mRNA deadenylation have been identified in yeast and higher eukaryotes. The mRNAs for transiently expressed proteins such as transcription factors, growth factors, cytokines and proto-oncogenes usually contain AU-rich elements (AREs) in the 3-UTR. These AREs are characterized by the presence of one or multiple copies of the pentanucleotide AUUUA and a high U content. For example, class I AREs contain one to three copies of AUUUA repeats embedded in U-rich sequences and are present in early response genes (*c-fos*, *c-myc*) and cytokine encoding genes. Class II AREs contain five to eight AUUUA copies and are only found in cytokine mRNAs (*GM-CSF*, *TNF-β* and *IL-3*). Class III AREs contain a long U-rich sequence instead of AUUUA repeats and these elements are found in the *c-jun* proto-oncogene and β-adrenergic receptor mRNAs.

cis-acting destabilizing elements have also found in the coding regions in the mammalian *c-fos* (Wellington et al., 1993; Schiavi et al., 1994), *c-myc* (Wisdom and Lee, 1991), *Drosophila fushi tarazu* (Ito and Jacobs-Lorena, 2001) and yeast *MATα1* transcripts (Shyu et al., 1991; Caponigro and Parker, 1996)). The function of these elements requires movement of the ribosome across the RNA harboring them. For example, in the yeast *MATα1* transcript, the ability of a 32 nucleotide region to stimulate deadenylation and degradation is stimulated by a region of rare codons just 5' to this 32 nucleotide suggesting that the presence of paused ribosome is required for this element to function (Parker and Jacobson, 1990; Caponigro et al., 1993).

3.2. *trans*-acting factors

It has been increasingly clear that factors involved in controlling mRNA degradation processes include a variety of mRNA binding proteins. The Pumilio-homology domain-protein families (PUF) of RNA-binding proteins have been shown to bind 3-UTR sequences and accelerate the targeted mRNA degradation (Zamore et al., 1997). In yeast, PUF3 is the first protein that has been shown to promote deadenylation of *COX17* transcript by binding its 3-UTR (Olivas and Parker, 2000). PUF3 does not affect translation but interacts directly with the mRNA degradation machinery. Hence, PUF-proteins, as specific regulators of mRNA deadenylation, have been conserved throughout eukaryotes (Olivas and Parker, 2000). Another yeast PUF protein, PUF5, inhibits the expression of the *HO* gene by promoting the decay of the *HO* mRNA through binding its 3'-UTR (Tadauchi et al., 2001). Recently, a genome-wide microarray analysis identified yeast PUF mRNA targets that are functionally related (Gerber et al., 2004).

4. CCR4-NOT complex, the deadenylation machinery in yeast

The evolutionarily conserved CCR4-NOT protein complex was initially thought to be a general transcriptional regulatory complex involving from transcription initiation to termination (Collart, 2003; Denis and Chen, 2003). The components of 1.0-MDa core complex were identified as CCR4, CAF1, NOT1 through 5, CAF40 and CAF130. Interestingly, it has recently been reported that CCR4-NOT complex is also involved in mRNA deadenylation, the first step of mRNA decay. Of those nine components, CCR4 and CAF1 were identified as the major deadenylase complex and when inactivated,

intracellular deadenylation processes were severely damaged (Tucker et al., 2001 & 2002; Chen et al., 2002).

4.1. CCR4

Yeast CCR4 protein has sequence homology to a family of Mg^{2+}-dependent nucleases related to *E. coli* exonuclease III in its C-terminal domain. Deletion of the CCR4 gene causes a decrease in the rate of deadenylation and a concomitant stabilization of mRNAs (Tucker et al., 2001). Point mutations in highly conserved key catalytic amino acids cause a similar deadenylation defect, demonstrating that the catalytic activity of the protein is required for deadenylation (Chen et al., 2002; Tucker et al., 2002). *Drosophila ccr4* mutants also have a deadenylation defect (Temme et al., 2004). Both yeast CCR4 and it mammalian homolog are active as poly(A)-specific 3'-exoribonuclease *in vitro* in the absence of the other subunits of the CCR4-NOT complex (Chen et al., 2002; Viswanathan et al., 2003). In addition to it C-terminal nuclease domain, CCR4 contains a conserved leucine-rich repeat domain which is essential for binding the CAF1 and also affects exonuclease activity (Clark et al., 2004).

4.2. CAF1

Yeast CAF1 (yCAF1) is a 49-kDa protein and a member of the RNase D family of exonuclease (Daugeron et al., 2001; Moser et al., 1997). It has been reported that the C-terminal ribonuclease domain of yeast CAF1 overexpressed and purified from *E. coli* displayed deadenylase activity with some poly(A) preference (Daugeron et al., 2001; Thore et al., 2003). Deletion of yeast *CAF1* gene leads to a similar deadenylation defect as a *ccr4*Δ mutation (Daugeron et al., 2001; Tucker et al., 2001). Mouse CAF1

(mCAF1) also display deadenylase activity and mutation of putative key, catalytic residues in the RNase D domain inactivates mCAF1 activity (Viswanathan et al., 2004). Similarly, inactivation of Drosophila CAF1 causes an increased steady-state poly(A) tail and a decrease in the rate of *HSP70* mRNA deadenylation (Temme et al., 2004).

5. Aim of this dissertation

The aim of this dissertation is to characterize the functional roles of yCAF1 in deadenylation and other possible processes. While yCAF1 is known to be the major component of deadenylase complex, its overall function to the deadenylation has not been characterized well. More importantly, our previous *in vitro* and *in vivo* experiments concerning deadenylase function of yCAF1 showed that it may not contain deadenylase activity. Several previous observations also support our observations. First, the catalytic domain of yCAF1 is not well conserved (Moser et al., 1997). Second, although yeast strains completely lacking CAF1 show a defect in deadenylation, strains containing a full-length but catalytically inactive CAF1 show no phenotype (Tucker et al., 2002; Chen et al., 2002; Thore et al., 2003). Third, *in vitro* deadenylase activity of yCAF1 is CCR4 dependent (Tucker et al., 2001). Fourth, overexpression of CCR4 can rescue the deadenylation defect of *caf1*, but overexpression of CAF1 does not compensate for that of *ccr4* (Tucker et al., 2001). Since deadenylation is the first rate-limiting step of mRNA degradation, the dissection of the functional properties of CAF1 will shed light on the mechanisms by which the rate of mRNA decay is modulated.

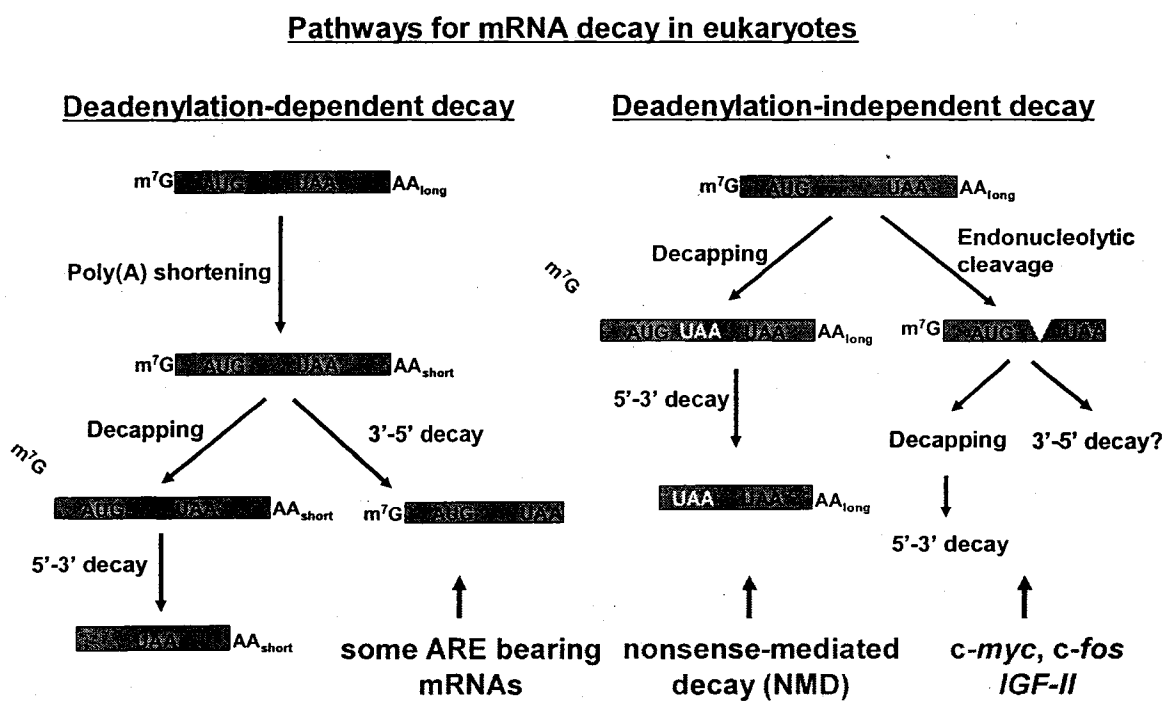


Figure 1. Eukaryotic mRNA decay pathways (modified from Beelman and Parker, 1995). Deadenylation-dependent decay pathway can lead to decapping and 5'-3' exonucleolytic cleavage or to 3' to 5' decay mediated by exosome. All eukaryotic mRNAs may be subjected to this pathway unless they are targeted for rapid deadenylation-independent decay pathways by a early stop codon. After deadenylation-independent decapping, mRNA is degraded in a 5' to 3' direction. Endonucleolytic cleavage of mRNA may serve as a one-step deadenylation that leads to decapping and 5' to 3' decay and, *c-myc*, *c-fos*, and *IGF-II* are known to be subjected to this pathway.

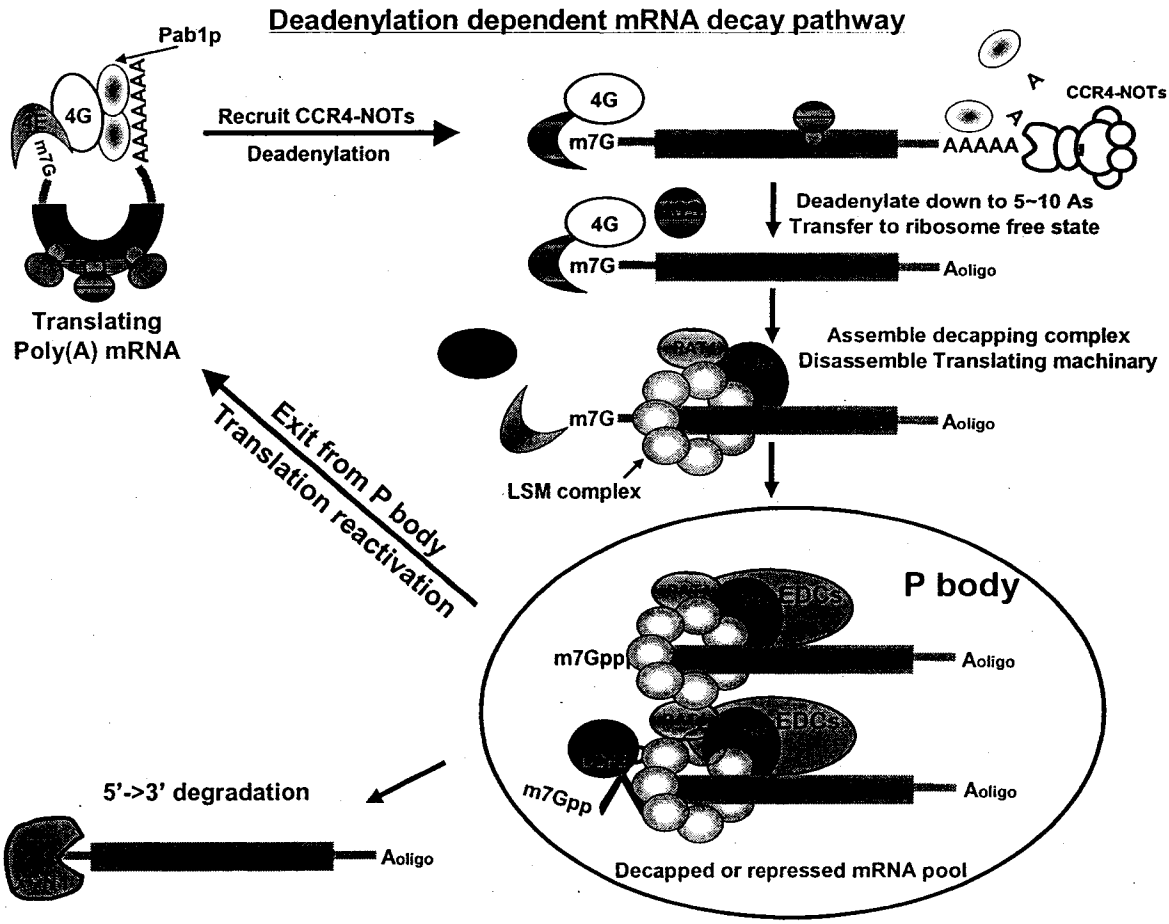


Figure 2. Detailed diagram of deadenylation dependent decay pathway (modified from Coller and Parker, 2004).

CHAPTER I

FUNCTIONAL ANALYSIS OF CAF1 PROTEIN

Introduction

In eukaryotes the major pathway of mRNA degradation is initiated by poly(A) tail shortening (deadenylation) followed by removal of the 5' cap structure (decapping) and 5' to 3' exonucleolytic cleavage of the mRNA body by the XRN1 exonuclease (Parker and Song, 2004; Beelman and Parker, 1995; Wilusz et al., 2001). Deadenylation as compared to decapping and 5'-3' nuclease digestion has been shown to be most important in the control of mRNA degradation rates (Cao and Parker, 2001). The CCR4-NOT complex in responsible for the majority of the poly(A) shortening process in yeast (Tucker et al., 2002 & 2001; Chen et al., 2002), and the 1.0 MDa core complex is comprised of ten components: CCR4, CAF1 (also known as POP2), five NOT proteins (NOT1-NOT5), CAF40, CAF130 and BTT1 (Liu et al., 1998; Bai et al., 1999; Chen et al., 2001; Denis and Chen, 2003). Of these proteins, CCR4 and CAF1 appear to be the most important players in the mRNA deadenylation process (Tucker et al., 2002 & 2001).

The CCR4 protein as a member of ExoIII family of nucleases/phosphatases (Dlakic, 2000; Dupressoir et al., 1999) is the catalytic subunit responsible for degradation of the mRNA poly(A) tail, and point mutations in the predicted active site of CCR4 cause deadenylation defects like that of a *ccr4* deletion (Chen et al., 2002; Tucker

et al., 2002). Similarly, a *ccr4* deletion mutant in *Drosophila* also displayed an in vivo deadenylation defect (Temme et al., 2004).

The role of the CAF1 protein, however, is less clear. Its principal role has been considered to be that of linking CCR4 to the remainder of the CCR4-NOT complex (Liu et al., 1998; Bai et al., 1999; Draper et al., 1995). Deletion of the *CAF1* gene showed a dramatic deadenylation defect in vivo, albeit to a lesser extent than that of *ccr4* (Tucker et al., 2001), and this has been interpreted to mean that CCR4 must be part of the CCR4-NOT complex to function well in vivo. Although CAF1 is classified as a member of the DEDDh family of nucleases (Moser et al., 1997; Zuo and Deutscher, 2001) and the polypeptide isolated from *E. coli* is active as a 3' to 5' exonuclease with some preference for poly(A) sequences, inactivation of predicted key catalytic active sites of CAF1 did not affect in vivo deadenylation function (Viswanathan et al., 2004). Moreover, overexpression of CCR4 could partially complement the deadenylation defect of a *caf1* deletion, but overexpression of CAF1 did not rescue phenotypes associated with that of a *ccr4* deletion (Tucker et al., 2001; Chen et al., 2002). While these results indicate that the deadenylase activity of CAF1 is not required for its in vivo deadenylation function, other evidence argues also against CAF1 functioning solely to link CCR4 to the remainder of the CCR4-NOT complex. For instance, defects in other NOT components do not result in deadenylation defects to nearly the same degree as that of a *caf1* deletion (Tucker et al., 2001). A *caf1* deletion is also lethal with deletion of the decapping/translational regulator *DHH1* (Coller and Parker, 2005), whereas a *ccr4* deletion is not (Maillet and Collart, 2002). Moreover, *caf1* has been shown to partially suppress translation stress effects in a manner similar to that of decapping defects whereas *ccr4* does not display this behavior (Holmes et al., 2004). These observations suggest roles of CAF1 in both deadenylation

and non-deadenylation processes.

Since the predicted catalytic sites of CAF1 are not critical to its in vivo function, understanding the functional roles of CAF1 on mRNA turnover will require the identification of other important regions of the CAF1 protein. To identify in the CAF1 protein functional regions which are critical for mRNA degradation, we conducted a deletion analysis of yeast CAF1 by targeting regions absolutely conserved among CAF1 orthologs. We demonstrate four important results in this report. First, we identify four functional regions of CAF1, including those required for binding CCR4 and for deadenylation. Second, we find that the domains of CAF1 that are most important for deadenylation in vivo are distinct from the region that is required for CCR4 binding or CAF1 catalysis, implying that CAF1 function is not primarily limited to simply linking CCR4 to the remainder of the CCR4-NOT complex. Third, defects in CAF1 but not that CCR4 appear to affect translation and to interact genetically with other factors controlling translation. Fourth, a *caf1* deletion when coupled with a PAB1 translation defect that does not by itself or when coupled with *ccr4* affect deadenylation severely reduces deadenylation. These results implicate CAF1 in communications with translation that also affect the deadenylation process.

Materials and Methods

Yeast strains and growth conditions.

The background yeast strains used in this study are listed in Table 1. Yeast strains were grown in YEP medium (2% yeast extract, 1% Bacto Peptone) or minimal medium supplemented with nutrients required for auxotrophic deficiencies and with 4% glucose or 2% galactose/2% raffinose unless otherwise indicated. YD plates consisted of YEP medium supplemented with 2% glucose and 2% agar and caffeine plates are YD plates with either 5, 8, or 15 mM caffeine.

Site-directed deletion mutagenesis.

The MPT0 plasmid harboring the yeast *CAF1* open reading frame was used as the template for the polymerase chain reaction (PCR). All PCR reactions were performed with Vent polymerase (New England Biolabs). In order to generate 173DVW175, 213FRS215, 255WQF257, 303SGL305, 329LMN331, and 340DFE342 deletions, we designed the following six pairs of oligonucleotide primers: caf1-1-f (5'-TATCTTTTCGTTCGCAAGTCCAACCTTTACAGTGAATTC-3') and caf1-1-r (5'-GTAAAGGTTGGACTTGCGAACGAAAAGATAATTTGGGG-3'), caf1-2-f (5'-AGGCCGATCGGCACTAAGGTCGATTACCACTATCAGACA-3') and caf1-2-r (5'-GTGGTAATCGACCTTAGTGCCGATCGGCCTAGCCAAAGT-3'), caf1-3-f (5'-AACGGTCCCTCAACGAATTTTGAATTTGACCCAAAGAAG-3') and caf1-3-r (5'-GTCAAATTCAAAATTCGTTGAGGGACCGTTGTCAGGCTT-3'), caf1-4-f (5'-CAGCTTCTAATGGACATGATGGATGATTCTGTTACTTGG-3') and caf1-4-r (5'-

AGAATCATCCATCATGTCCATTAGAAGCTGCGAAAATTC-3'), caf1-5-f (5'-TTCCTGATCAACATTGACTCCATGCCCAACAACAAGGAG-3') and caf1-5-r (5'-GTTGGGCATGGAGTCAATGTTGATCAGGAAACCTAGATC-3'), and caf1-6-f (5'-CCCAACAACAAGGAGTGGTGGGTCCATCAATACATGCCC-3') and caf1-6-r (5'-TTGATGGACCCACCACTCCTTGTTGTTGGGCATGGAGTC-3'). These primers are used for the first PCR reaction in combination with BamHI and HindIII restriction site introduced Caf1-Bm-f (5'-AAAGGATCCATGCAATCTATGAATGTACAA) and caf1/2291-Hind (5'-TACATATAAAGCTTAAATGATCATTGGTCCC-3') primers. The first PCR products were used for amplifying final full length *CAF1* mutant alleles using Caf1-Bm-f and caf1/2291-Hind primers. The final PCR products were purified, digested with BamHI and HindIII, and inserted into BamHI and HindIII- digested pET-23a(+) to generate plasmids pTB8a (*CAF1*), pTB8-1 (*caf1-1*), pTB8-2 (*caf1-2*), pTB8-3 (*caf1-3*), pTB8-4 (*caf1-4*), pTB8-5 (*caf1-5*), and pTB8-4 (*caf1-6*), respectively. All the sequences of the mutagenized *CAF1* alleles were verified by sequencing. For further analysis *CAF1* open reading frames were subcloned into pLexA202-2 (Cook et al., 1994), pGEX-KG, pJG4-5 (Cook et al., 1994), and pJCN112 (Chen et al., 2002) to make N-terminal LexA, GST, HA, and N-terminal Flag with C-terminal 6His epitope tagged CAF1 proteins, respectively.

Library screening

Strain 319-c1-lN (*caf1Δ pab1Δ*) carrying pYC360 (*PAB1 CEN URA3*) and pYC506 (*pab1-ΔRRM2 TRP1*) was transformed with a YEp13 (2μm *LEU2*) high-copy-number yeast genomic library and plated on synthetic drop-out media lacking uracil, leucine, and tryptophane for selecting transformants. Approximately 15,000 Leu^+

colonies were subsequently replica plated onto minimal media containing 5-fluoroorotic acid (5-FOA). The plasmids from yeast capable of growing on FOA plates were rescued, transformed into *E. coli* DH5α, and analyzed by colony PCR and restriction digestion to identify YEp13 based plasmids. The selected plasmids were retransformed into the original yeast strain to confirm the high-copy-number suppression of the *pab1-ΔRRM2 caf1* synthetic lethality. The yeast genome segments containing suppressor genes were identified by sequence analysis.

Flag pull-down analysis

The yeast cultures containing FLAG-CAF1 fusions were grown to late log phase in 50 ml selective media supplemented with 4% glucose, shifted to 100 ml of the same media with 2% glactose/raffinose and grown for 16 hours. The cells were washed and lysed in extraction buffer (50 mM Tris-Cl [pH7.9], 150 mM NaCl, 0.1 mM $MgCl_2$, 0.1% NP40, 20% glycerol) plus a protease inhibitor cocktail. After clarification of the crude cell lysate by centrifugation at 15,000 × g at 4°C for 15 min twice, the supernatants were incubated with 400 μl of anti-FLAG M2 affinity agarose (Sigma) at 4°C overnight. The bound resins were washed with 10 ml wash buffer (extraction buffer with 5% glycerol) three times, transferred to eppendorf tubes and washed with 1 ml wash buffer five times. The resultant precipitates were run subsequently on an 8% SDS-polyacrylamide gel, electro-transferred to polyvinylidene fluoride (PVDF) membrane (Immobilon™-P, Millipore) and analyzed by Western blot using SuperSignal® West Pico Luminol/Enhancer Solution (Pierce).

Protein extraction, *in vitro* deadenylation assay, and *in vivo* translation assays.

FLAG-CCR4 protein purification was performed as described previously with some modifications (Viswanathan et al., 2003). Briefly, the EGY188-c1-1a-1 cells harboring single copy of CCR4 (pYC343) and the LexA-CAF1 fusion variants were grown in 1 liter of selective medium with 4% glucose to mid-log phase (OD_{600} of 0.7). The cells were harvested, washed once with pure water and lysed in Buffer A (50 mM Tris-Cl [pH 7.9], 150 mM NaCl, 0.1 mM $MgCl_2$, 0.1% NP40, 10% glycerol) supplemented with protease inhibitors. After clarification of the crude lysate by centrifugation (15,000 × g at 4°C for 20 min), the supernatants were incubated with pre-equilibrated anti-FLAG M2 antibody-agarose (Sigma) at 4°C for 4 h. After washing of the FLAG agarose beads with 10 ml of washing buffer (bufferA with 5% glycerol) three times, the FLAG fusion proteins were eluted twice with buffer A containing 200 μg/ml FLAG peptide (Sigma). In vitro deadenylation assays were conducted as described (Viswanathan et al., 2003 & 2004). The rates of *in vivo* protein synthesis were determined by quantitating the amount of [^{35}S]-methionine incorporation into protein as described (Schwartz and Parker, 1999).

RNA preparation and analyses.

RNA samples were prepared using the hot acidic phenol method described in (Cook and Denis, 1993). Yeast cells were resuspended in TES (10 mM Tris-Cl [pH7.5], 10 mM EDTA, 0.5% SDS) and acid phenol and incubated at 65 °C for 45 min with frequent vortexing. The lysates were then purified with acid phenol and chloroform. Total RNA was then precipitated with alcohol and dissolved in DEPC water.

To perform the transcriptional pulse-chase experiments, strain EGY188-cl

carrying the individual LexA-caf1 variants was grown in 5 ml synthetic media supplemented with 2% raffinose, transferred and regrown in the 100 ml fresh media until mid-log phase (OD_{600} of 0.7). Cells were then harvested, washed once with fresh media and transferred to 15 ml of fresh media containing 2% raffinose and grown for 15 min. *GAL1* transcription was activated by adding galactose at a 2% final concentration for 15 min and shut off by adding glucose to a concentration of 4%. Since yeast cells harboring the LexA-caf1-2 protein resulted in poor *GAL1* transcription, the *GAL1* promoter was activated for 30 minutes prior to transcription shut off.

The deadenylation rates and end points for *GAL1* mRNA were determined following RNase H treatment of purified RNAs as described previously (Muhlrad and Parker, 1992). An oligonucleotide probe (5'-GCCATTTGGGCCCCCTGG-3) complementary to a segment 133 bp upstream of the *GAL1* translational stop codon was hybridized with 12~20 μg of total RNA prior to RNase H digestion. The resultant *GAL1* 3' polyadenylated species were separated on a denaturing polyacrylamide gel (6%/7.5 M urea) and detected by Northern analysis using a probe complementary to the 3' end of the *GAL1* mRNA (5'-GCCCAATGCTGGTTTAGAGACGATGATAGCATTTTCTAGCTCAGCATCAGTGATCTTAGGG-3). The rate of deadenylation was determined for the shortest poly(A) tail as previously described (Tucker et al., 2001; Viswanathan et al., 2004).

Table 1. List of yeast strains

Strain	Genotype
EGY188	*MAT*a *ura3 his3 trp1 LexA-LEU2*
EGY188-c1	Isogenic to EGY188 except *caf1*::*URA3*
EGY188-c1-1	Isogenic to EGY188 except *caf1*::*ura3*
EGY188-1a-1-c1	Isogenic to EGY188 except *ccr4*::*ura3 caf1*::*LEU2*
EGY191	*MAT*α *ura3 his3 trp1 LexA-LEU2*
EGY191-c1	Isogenic to EGY191 except *caf1*::*URA3*
KY803-c1	*MAT*a *leu2-PET56 trp1-Δ1 ura3-52 gal2 gcn4-Δ1 caf1*::*LEU2*
AS319/YC504	*MAT*α *ura3leu2 his3 trp1pab1*::*HIS3* [PAB1-TRP1]
AS319-1a-uN/YC504	Isogenic to AS319 except *ccr4*::*ura3*::*NEO*
AS319-c1-lN	Isogenic to AS319 except *caf1*::*leu2*::*NEO*
AS319-cl-lN/YC505	Isogenic to AS319-cl-lN except [PAB1-ΔRRM1-TRP1] [PAB1-URA3]
AS319-cl-lN/YC506	Isogenic to AS319-cl-lN except [PAB1-ΔRRM2-TRP1] [PAB1-URA3]
AS319-d1-uL/YC504	Isogenic to AS319 except *dhh1*::*ura3*::*LEU2*
1881/YC504	Isogenic to AS319 except *cdc33-1*

Results

Purified yeast CAF1 does not display RNase function *in vitro*

It has been reported that C-terminal exonuclease domain of yeast CAF1 purified from *E. coli* showed deadenylase function in vitro with a poly(A) preference (Thore et al., 2003). In order to verify the enzymatic function of the CAF1 protein, we constructed a full length CAF1 with a FLAG tag at its N-terminal end. Upon transformation into a *ccr4 caf1* double deletion strain, we purified FLAG-CAF1 fusion protein utilizing a one-step affinity chromatography. Due to a strong interaction with CCR4, which has been verified as the major deadenylase, we had to use *ccr4 caf1* double knockout strain in order to avoid copurifying CCR4 enzymatic activity. Purified FLAG-CAF1 protein sample was then assayed for it enzymatic activities on a 25N+20A synthetic ssRNA substrates.

As shown in Figure 3, purified CCR4-FLAG displayed strong deadenylase activity as reported (Chen et al., 2002). In contrast, purified FLAG-CAF1 failed to function as a deadenylase. The ccr4-1-FLAG mutant protein (E556A) mutated in a key catalytic amino acid in exonuclease domain was used as a control and failed to function as deadenylase as expected (Chen et al., 2002).

The deadenylase function of yeast CAF1 is not required *in vivo*

Although yeast CAF1 has been shown to display deadenylase functions in vitro, this deadenylase function of CAF1 could not be assessed by our *in vitro* enzyme assay with purified N-terminal FLAG tagged CAF1 protein from *ccr4 caf1* double knock-out strain. We determined subsequently whether the putative enzyme activities of yeast CAF1 were required *in vivo*. LexA-yCAF1 proteins were created containing either the

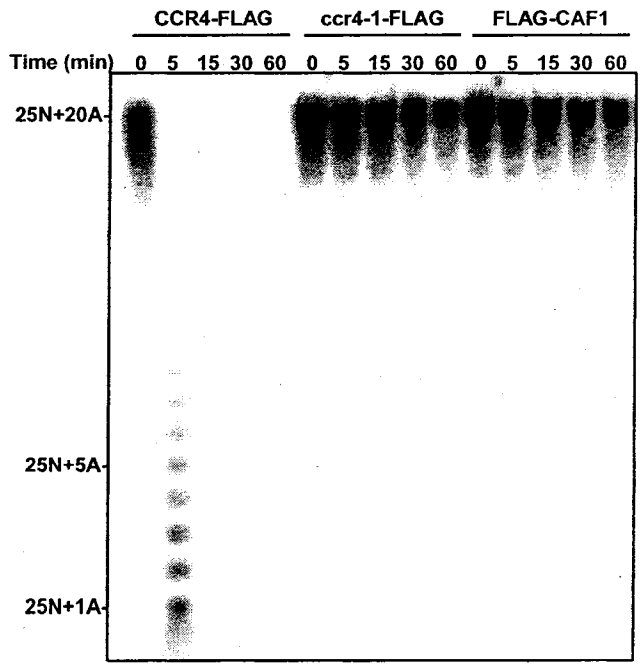


Figure 3. Yeast CAF1 does not display deadenylase activity in vitro. N-terminal FLAG tagged yeast CAF1 proteins are overexpressed and purified from *ccr4 caf1* double knock-out strain (see "Materials and Methods"). Purified CCR4-FLAG and ccr4-1-FLAG proteins were used as the positive and negative control, respectively. Radiolabeled 25N+20A RNA substrates were incubated with equivalent amounts of the three proteins at time zero for the times indicated.

S199A/E201A alteration that inactivated yCAF1 deadenylase function in vitro (Thore et al., 2003), which is comparable to the D39A/E41A mutation in mCAF1 that blocked its deadenylase activity. The expression of LexA-yCAF1 (S199A/E201A) and LexA-yCAF1 (Y320A/D321A) completely complemented the caffeine sensitivity of the yeast *caf1* deletion as did a LexA-yCAF1 protein (Viswanathan et al., 2004), suggesting that the enzymatic function of CAF1 is not required for its in vivo function.

We additionally analyzed the ability of LexA-yCAF1 (S199A/E201A) to allow deadenylation of the *GAL1* mRNAs in vivo. *GAL1* mRNA is polyadenylated at two locations 110 nt apart (Miyajima et al., 1984; Cui and Denis, 2003). Following the induction of *GAL1* mRNA synthesis for 12 min with galactose and repression at time zero with glucose, the rate of deadenylation of the two *GAL1* mRNAs was followed in a *caf1* strain containing LexA-yCAF1, LexA-yCAF1 (S199A/E201A), or LexA. LexA-yCAF1 allowed an efficient deadenylation of both *GAL1* long and *GAL1* short mRNA (Fig. 4), and CCR4 deadenylated the two mRNAs at rates of 4.7 and 3.4 A's/min, respectively. In contrast, in a *caf1* background expressing only LexA, deadenylation was slowed for both mRNA (rates of 1.9 and 1.3 A's/min, respectively). Incomplete deadenylation was also observed at late times in the *caf1* background (Figure 4) in agreement with previous observations that *caf1* causes a deadenylation end-point defect (Tucker et al., 2001). In contrast, the *caf1*/LexA-yCAF1 (S199A/E201A) strain deadenylated both *GAL1* mRNAs at comparable rates and to similar extents as that observed with LexA-yCAF1 (Fig. 4, rate of deadenylation for *GAL1* long was 4.4 A's/min and for *GAL1* short was 2.7 A's/min). These data indicate that the yCAF1 deadenylase function is not required in vivo in the deadenylation process.

We also tested whether the deadenylase function of mCAF1 the deadenylase

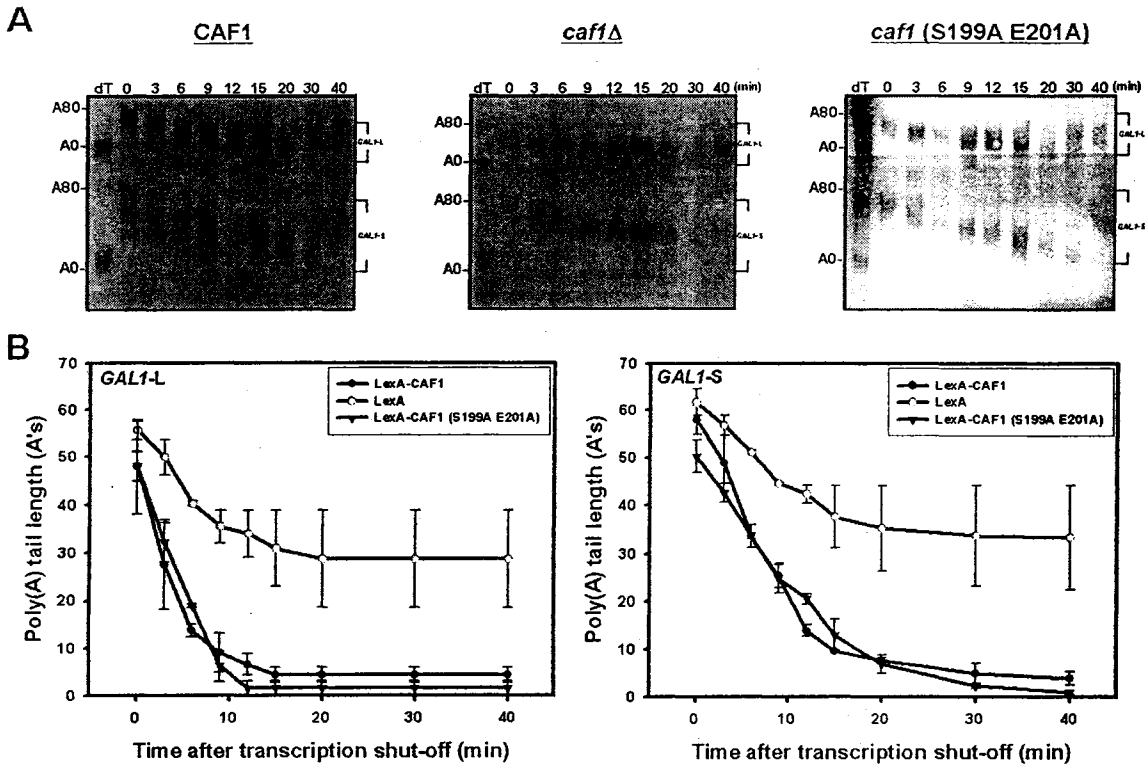


Figure 4. Mutations that inactivate yCAF1 deadenylase function in vitro do not affect deadenylation of *GAL1* in vivo. A, strain EGY188-c1 expressing LexA-yCAF1, LexA-yCAF1 (S199A/E201A), and LexA was subjected to pulse-chase analysis to determine the rates at which GAL1 mRNA deadenylated in vivo. Following an 12-min induction of the *GAL1* gene with galactose, its transcription was shut off at time zero with glucose, and RNA samples were taken at the times indicated. dT refers to the addition of oligo (dT) and RNase H to remove the poly(A) tail. B, the graphical summary of the average 2~3 determinations is represented with ± S.E.M. as indicated.

function of mCAF1 was required for complementing a *caf1* deletion. A yCAF1/mCAF1 fusion (carrying the unique N-terminal of yCAF1 and the DEDDh domain of mCAF1) was used for this analysis, because it gives better complementation than does mCAF1 alone (Shimizu-Yoshida et al., 1999). We found that mCAF1 mutations (D39A/E41A and Y159A/D160A), which inactivated mCAF1 in vitro, like similar mutations in yCAF1, did not interfere with the ability of yCAF1/mCAF1 hybrid protein to complement the caffeine sensitivity of a *caf1* deletion (Viswanathan et al., 2004).

Mutagenesis of CAF1 in the regions that are highly conserved among CAF1 orthologs

In order to identify CAF1-specific functional regions we targeted for mutagenesis the regions of CAF1 that are common only to CAF1 orthologs and are not present in any other DEDDh nuclease family members (Zuo and Deutscher, 2001; Moser et al., 1997). Six regions were identified as absolutely conserved among CAF1 orthologs (Figure 5B). Three amino acid deletions were made in each case to remove the region of homology, resulting in *caf1* alleles 1 through 6. After these deletions were constructed and their phenotypes were obtained, the X-ray crystallographic analysis of the C-terminal RNase D domain of CAF1 protein was published (Thore et al., 2003). The relative locations of each of the constructed *caf1* alleles are shown in Figure 5A: *caf1-1* (173DVW175) and *caf1-3* (255WQF257) alleles are positioned in β-sheets β1 and β3 that hydrogen bond to each, *caf1-2* (213FRS215) is located between β2 and α3 in a crystallographically undefined loop region, and *caf1-4* (303SGL305), *caf1-5* (329LMN331), and *caf1-6* (340DFE342), are located between α6 and β5, between α7 and α8, and on α8, respectively.

Each of the *caf1* mutant alleles was subsequently cloned into various kinds of

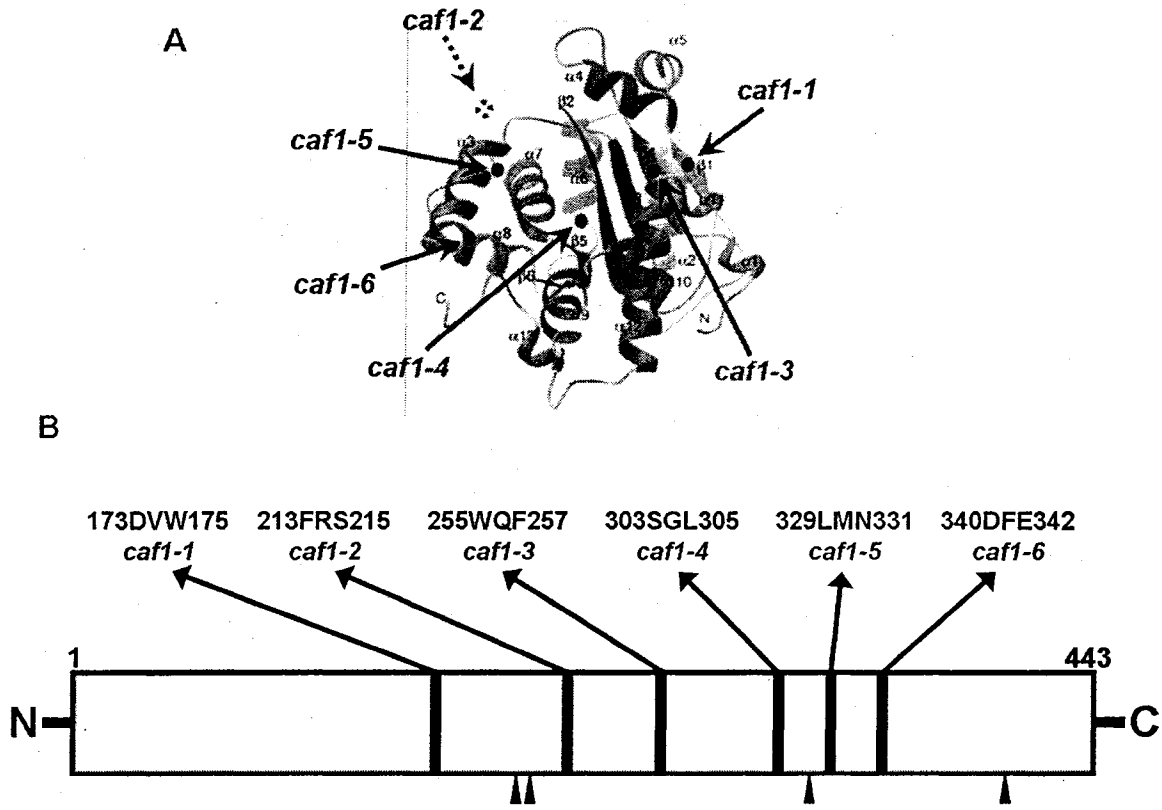


Figure 5. Mutagenesis of CAF1.

(A) Location of *CAF1* mutations are illustrated on the three-dimensional structure of the nuclease domain of the CAF1 protein (Thore et al., 2003).

(B) The six absolutely conserved regions targeted for mutagenesis are composed of three amino acids each as indicated above the schematic representation of CAF1 and are located in regions separate from the putative four key catalytic amino acids conserved among DEDDh ribonucleases (black arrows). The names of the mutant alleles are also shown beneath the deleted amino acids.

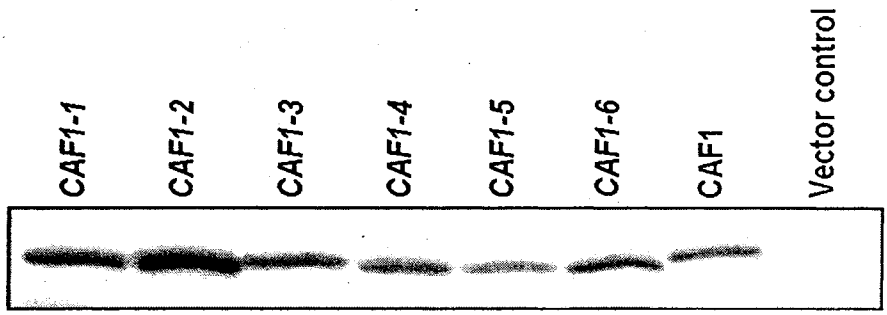


Figure 6. Western blot analysis of N-terminal HA epitope tagging CAF1 mutant proteins. The HA-CAF1 proteins were expressed in the EGY191-c1 strain, extracted and subjected to Western analysis. The apparent increased abundance of caf1-2 protein in the gel that is presented is artifactual and no significant differences in the abundance of these proteins was observed in other experiments (see also Figure 7).

plasmid vectors such as pLexA(202), pJG4-5 (HA-tagged), and pJCN112 (FLAG-tagged) for further analysis (see Materials and Methods). Each of the mutant proteins was expressed to comparable levels as that found for the wild-type CAF1 protein, indicating that these small deletions did not affect the protein expression or stability of CAF1 (Figure 6; data not shown).

caf1-1, -3, and -6 alleles display similar phenotypes to a *caf1* null allele

One of the most prominent phenotypes of a strain carrying a *caf1* deletion is its caffeine sensitivity, although other phenotypes such as temperature sensitivity and slow growth have been observed (Sakai et al., 1992; Draper et al., 1995; Hata et al., 1998). To examine the phenotypes of *CAF1* mutant alleles, we tested the growth phenotypes of the *caf1* alleles by using the EGY188-c1 (*caf1*) strain carrying a plasmid expressing an N-terminal LexA fusion with each of the mutant CAF1 proteins (Table 2). Of the six *CAF1* mutants, *caf1-1*, *caf1-3* and *caf1-6* alleles resulted in an inability to grow on YD plates containing 8 mM caffeine. Interestingly, the *caf1-1* and *caf1-3* alleles showed a dominant negative growth defect as their growth rate was observed to be slowed as compared to that of the *caf1* null allele (data not shown). The *caf1-2* and *caf1-5* alleles displayed only weak sensitivity to caffeine, and for the *caf1-4* allele, we did not observe any difference in growth on caffeine plates as compared to the wild-type strain (Table 2).

Wild-type, *caf1-4*, and *caf1-5* alleles were able to grow partially at 41°C (Table 2), whereas the *caf1-1*, *-2*, *-3*, and *-6* alleles, like the *caf1* deletion, failed to grow at 41°C. In order to verify that the three amino acid deletions we constructed did not dramatically affect the structure of the protein and therefore were the cause for the caffeine and temperature sensitivity phenotypes that were observed, we constructed two additional

Table 2. Growth phenotype test of yeast cells carrying *caf1* mutant alleles

CAF1 allele	8 mM caffeine	41 °C
Wild-type	++	**w**
caf1-1	–	–
caf1-2	**w**	–
caf1-3	–	–
caf1-4	++	**w**
caf1-5	+	**w**
caf1-6	–	–
caf1Δ	–	–
caf1-10	**w**	–
caf1-11	–	–

EGY188-c1 strains carrying LexA-CAF1 proteins or pLexA(202) vector alone (*caf1Δ*) were tested. Growth was detected on YEP plates containing 4% glucose (YD) and supplemented with 8 mM caffeine. Temperature sensitive growth was monitored on YD plates at 41°C. ++, strong growth; +, good growth; w, weak growth; –, no growth.

caf1 alleles in which three alanine residues were substituted for the three residues deleted in *caf1-2* and *caf1-3*. These two alleles were chosen in that results shown below indicated that the *caf1-1* and *caf1-6* alleles appeared phenotypically similar to *caf1-3*, and *caf1-2* was unique for being defective in CCR4 binding. *caf1-10* (213AAA215) and *caf1-11* (255AAA257) displayed the exact same phenotypes as their respective deletion alleles, *caf1-2* and *caf1-3* (Table 2). For the remainder of our analysis the original deletion mutations were used.

The caf1-2 protein is defective in binding with CCR4

The relative locations of the proteins in the CCR4-NOT core complex have been determined (Bai et al., 1999; Chen et al., 2001; Denis and Chen, 2003) in which the CAF1 protein is critical for linking CCR4 to the remainder of the CCR4-NOT complex. We, therefore, assessed the ability of each of the CAF1 mutant proteins to bind CCR4, using FLAG-tagged CAF1 protein variants expressed in a *caf1* deletion background. After FLAG immunoprecipitations were conducted, as shown in Figure 7, we found that the *caf1-4* protein was able to bind CCR4 as well as wild-type CAF1. The *caf1-2* protein, in contrast, was totally defective in binding to CCR4 (Figure 7). While the caf1-1, -3, -5, and -6 proteins displayed reduced binding to CCR4, they still displayed significant association with CCR4. All of the mutant proteins were still capable of binding NOT1, although they did appear to display reduced binding to NOT1 as compared to wild-type. These results suggest several conclusions. First, they indicate that the differences in the growth and caffeine phenotypes described above do not result from differences in CAF1 binding NOT1. Second, and most importantly, the severe phenotypes displayed with the *caf1-1*, *-3*, and *-6* alleles was not due to loss of CCR4 binding, as each of the proteins

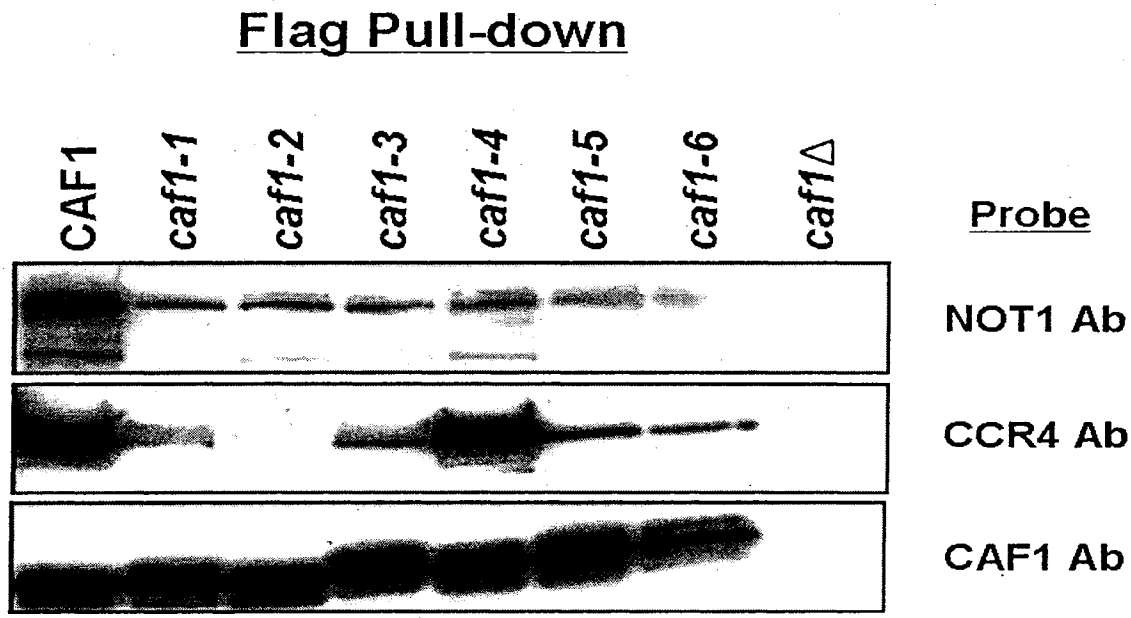


Figure 7. *caf1-2* fails to contact CCR4 *in vivo*. N-terminal FLAG tagged *CAF1* alleles were transformed into a *caf1* deletion strain, KY803-c1. Crude extracts (5 mg) were immunoprecipitated with anti-FLAG M2 antibody and proteins were visualized by Western blot analysis with antibodies directed against CCR4, NOT1, and CAF1 as indicated.

could bind CCR4 as well as *caf1-5* which did not display as severe growth defects. Third, the caf1-2 protein was completely defective for binding CCR4 and yet the *caf1-2* strain grew better than did *caf1-1*, *-3*, or *-6*, indicating that loss of CCR4 binding alone was not the cause for the *caf1* deletion phenotypes. Fourth, the ratio of NOT1 to CCR4 in the *caf1-1*, *-3*, *-5*, and *-6* immunoprecipitations was similar to that found in the wild-type strain, arguing that the CCR4-NOT complex remained intact, albeit probably less abundant in the presence of these *caf1* alleles.

The ability of CAF1 to bind CCR4 is not required for CCR4 deadenylation function *in vitro*

Immunoprecipitation studies showed that *caf1-2* is totally defective in binding with CCR4 and the remaining *caf1* mutants showed that they were still capable of binding to CCR4. In order to identity if the interaction between CCR4 and CAF1 affects the CCR4 deadenylation function in vitro, we performed enzyme assays with radiolabeled 25N+20A synthetic RNA substrate (Figure 8A). For this experiment, N-terminal FLAG tagged single copy *CCR4* and *CAF1* mutant alleles were transformed into a *ccr4Δ caf1Δ* background. CCR4 proteins were subsequently purified by using affinity chromatography and equivalent amounts of CCR4 were used in the *in vitro* deadenylation reactions (Figure 8B). The relative rate of deadenylation from each reaction were quantified by using Phosphorimager software and normalized by setting wild-type reaction to 100. As shown in Figure 8 and Table 3, there is no effect of the *caf1* alleles on in vitro CCR4 deadenylation. For example, CCR4 from *caf1-2*, showed almost no difference in deadenylation function as compared to CCR4 purified from wild-type. While CCR4 from *caf1-1*, *caf1-3*, and *caf1-6* that still have residual binding function to

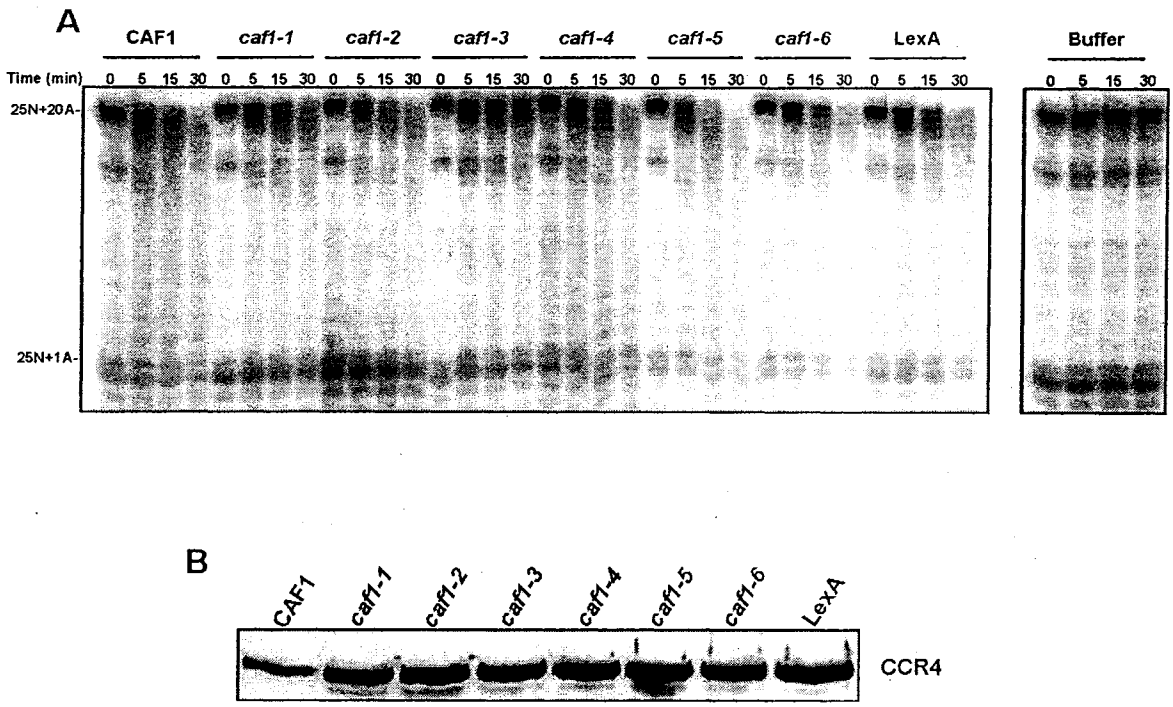


Figure 8. CAF1 binding to CCR4 does not affect the deadenylase function of CCR4 in vitro. (A) Deadenylation assays with single copy FLAG-CCR4 fusion protein purified from the yeast cells containing *caf1* mutant alleles. Radioactively labeled synthetic 25N+20A RNA substrates were incubated with purified CCR4 protein and removed at each time point. The removed samples were subsequently separated on a 15% polyacrylamide, 8 M urea gel and visualized by a PhosphoImager. The length of the RNA substrate is indicated on the left. (B) Purified FLAG-CCR4 proteins from each of the *caf1* mutants were visualized by Western blot analysis. Equivalent amounts of FLAG-CCR4 protein were used in deadenylation assay (A).

Table 3. Relative deadenylation rates of CCR4 isolated from *caf1* mutant alleles

CAF1 alleles	Relative deadenylation rate
CAF1	100
caf1-1	88
caf1-2	93
caf1-3	69
caf1-4	91
caf1-5	135
caf1-6	73
caf1Δ	80

[^{32}P] labeled 25N+20A synthetic RNAs were used as the substrate. The reaction products were subjected to polyacrylamide denaturing gel electrophoresis as described in Figure 8 and the Materials and Methods, and analyzed and quantified by a Phosphor imager and its software (Bio-Rad). The deadenylation rate of CCR4 from the wild-type strain was set at 100.

CCR4 (Figure 3), they displayed about the same activity as compared to *caf1-2*. This result suggests that, at least *in vitro*, the CCR4 and CAF1 interaction is not necessary in the deadenylation process and the dominant negative *in vivo* phenotype showed with *caf1-1* is not due to a poisoning of CCR4 deadenylation activity.

Identification of separable functional regions required for mRNA deadenylation

In order to determine if the *caf1* mutant alleles affected mRNA deadenylation in vivo, transcriptional pulse-chase experiments were performed on the *GAL1* mRNA. In this experiment, transcription of *GAL1* mRNA was induced by adding galactose and then repressed by adding glucose to the growing culture. Following a 15 minute induction, *GAL1* mRNA synthesis was shut off at time zero and aliquots were prepared for Northern analysis at each time point. As shown in Figure 9A, *GAL1* mRNA was shortened with a 3' oligonucleotide probe and RNase H prior to separation on a denaturing polyacrylamide gel for size resolution of the poly(A) tail. Due to the two polyadenylation sites in the *GAL1* gene, long and short *GAL1* mRNAs (*GAL1*-L & -S) can be analyzed simultaneously (Miyajima et al., 1984; Cui and Denis, 2003). The *GAL1*-L has an additional 110 nt in its 3'-UTR segment as compared to *GAL1*-S (Figure 9A). These two mRNA, also display different rates of deadenylation (Cui and Denis, 2003), allowing us to analyze the effect of the *caf1* alleles on differentially deadenylated mRNAs. For this experiment LexA fusions to the CAF1 variants were used to transform a *caf1* deletion yeast strain.

The deadenylation rate and endpoint analysis of *GAL1* mRNA identified three functional regions that are important for normal deadenylation in vivo. Of the six *caf1* alleles, *caf1-1*, *caf1-3*, and *caf1-6* alleles displayed severe deadenylation defects similar

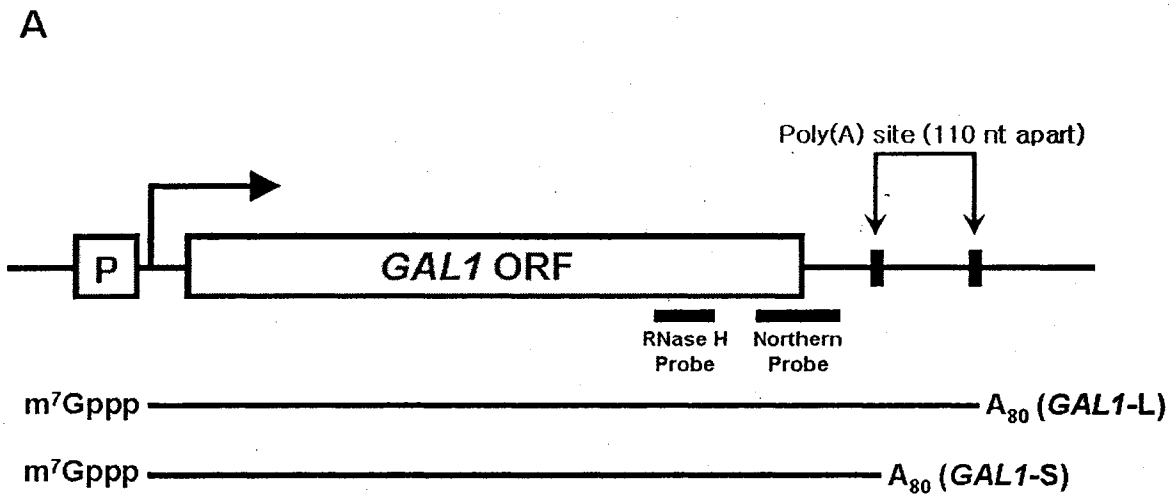


Figure 9. Mutagenesis of CAF1 identifies separable functional regions involved in deadenylation.

(A): Diagram for the *GAL1* gene and its two mRNAs.

(B): Transcriptional pulse-chase analyses on *GAL1* mRNA were conducted with EGY188-c1 harboring each LexA-*caf1* fusion or the LexA vector as the negative control. Following a 15 to 30-min induction of the *GAL1* gene by addition of galactose, transcription was shut off at time zero by adding glucose. Northern blot analyses were conducted with RNA samples prepared from yeast cells harvested at the time points indicated. dT refers to the RNA sample probed with oligo (dT) followed by RNase H digestion to remove the poly(A) tail.

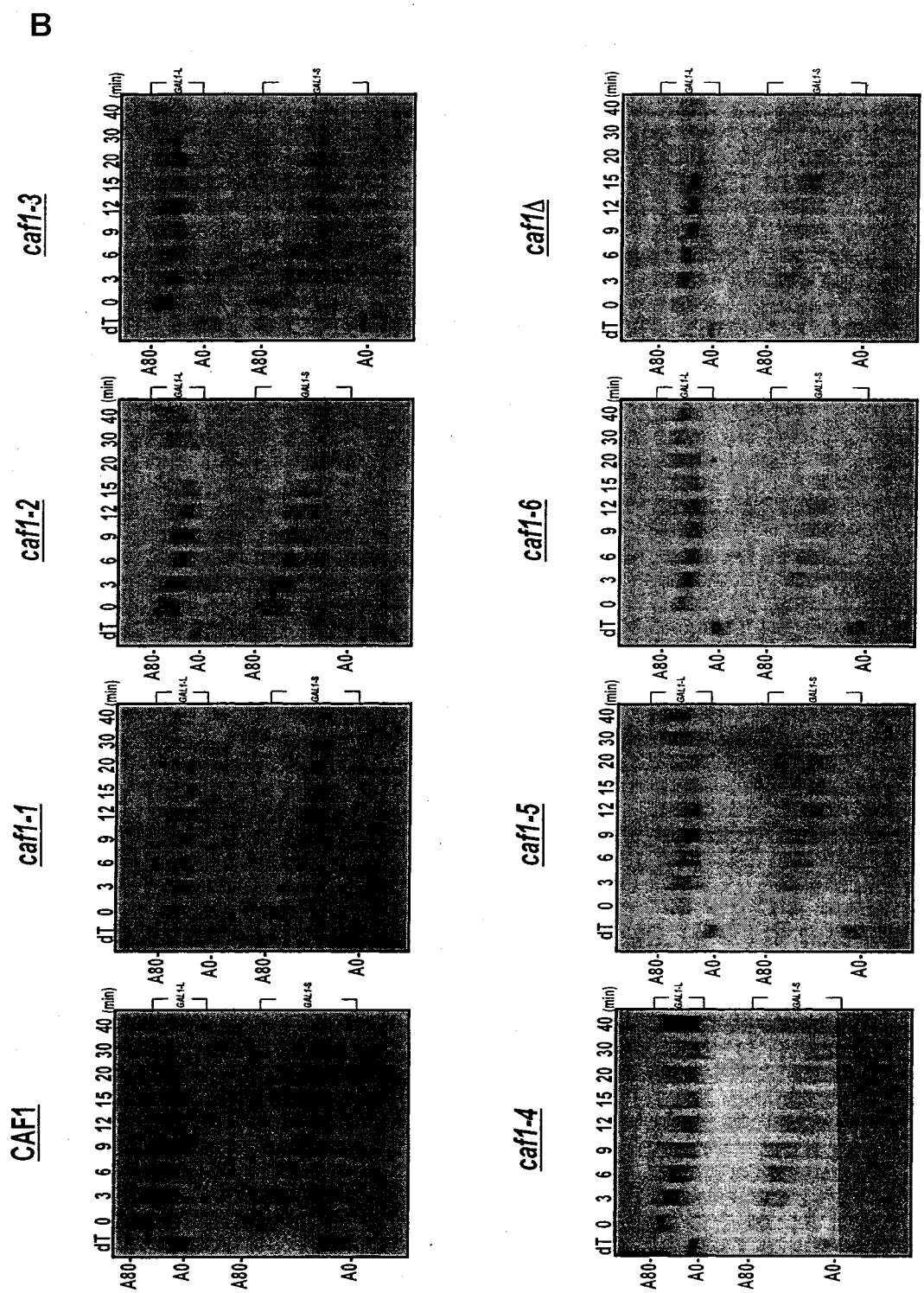


Table 4. Deadenylation rates of *GAL1* and endpoints of *MFA2pG* mRNA

CAF1 allele	Deadenylation Rate (A's/min±SEM)		Deadenylation endpoint (oligo(A)±SEM)
	GAL1-L	*GAL1*-S	*MFA2pG*
Wild-type	4.3±0.7	3.6±0.2	11±1.9
caf1-1	1.7±0.1	2.5±0.05	29±3.4
caf1-2	3.4±0.7	2.8±0.04	15±2.1
caf1-3	1.8±0.2	2.2±0.5	29±3.4
caf1-4	5.0±0.5	4.4±2.0	11±2.1
caf1-5	3.6±0.2	2.2±0.5	12±4.3
caf1-6	2.4±0.2	2.7±0.5	24±2.0
*caf1*Δ	2.4±0.5	1.9±0.3	26±2.7

caf1 deletion strains (EGY188-c1) harboring LexA version of wild-type, *caf1* mutants, and vector-self (pLexA202-2) were used for transcriptional pulse chase analysis. Rates of deadenylation were measured by the change in the length of the shortest poly(A) tail as a function of time following transcriptional shut-off (Figure 4B). EGY188-c1-1 strains harboring LexA-caf1 variants and pRP485 (GAL1-*MFA2pG*) were used for analyzing steady state *MFA2pG* mRNA. Longest *pG* fragments from Northern analysis were taken for deadenylation endpoint measurements. All values represent the average of three separate experiments and the standard error of the mean (SEM).

to that displayed with a *caf1* null allele. For the wild-type strain (Figure 9B), *GAL1*-L poly(A) tails were shortened to 8-12 nt within 9 min and for those of *GAL1*-S in 12 min. In contrast, the *caf1-1*, *-3*, and *-6* alleles failed to deadenylate completely even after 40 min for both *GAL1*-L and -S (Figure 9B). The average rates of deadenylation for *GAL1*-L and *GAL1*-S inwild-type were 4.3 and 3.6 A's/min, respectively (Table 4). In the *caf1-1*, *-3*, and *-6* mutated strains, the range of the deadenylation rates for the long and short *GAL1* mRNAs were from 1.7 to 2.4 and from 2.2 to 2.7 nucleotides per minute, respectively (Table 4). For *caf1-2* and *caf1-5*, in contrast, the deadenylation rates for *GAL1*-L were more similar to wild-type cells (Table 4; Figure 9B). However, *caf1-2* and *caf1-5* displayed greater effects on the deadenylation rate for *GAL1*-S, suggesting that they may be defective in deadenylation for a subset of mRNA species. Given that the phenotypic analysis of *caf1-2* and *caf1-5* showed that their caffeine sensitivities were halfway between wild-type and the *caf1* deletion, these in vivo deadenylation data agree well with their in vivo phenotypes.

Steady state *MFA2pG* mRNA analysis in *caf1* alleles

Deadenylation endpoint analysis of steady state RNA levels of *MFA2pG* was also conducted as *caf1* has previously been shown to result in endpoints of about 16-20 A's as compared to 8-12 A's for wild-type *CAF1* (Tucker et al., 2001). We found that *caf1-1*, *-3*, and *-6* alleles resulted in the longer poly(A) tail length for the *pG* fragment that results after deadenylation/ decapping/ and 5'-3' nuclease action on *MFA2pG* than for wild-type CAF1: 24-29 A's as compared to 11 A's (Figure 10, lanes 2, 4, 7 compared to lane 1; summarized in Table 4). In the *caf1-2* and *caf1-5* alleles, the deadenylation endpoints of the *pG* fragment were not significantly affected (lanes 3 and 6). These

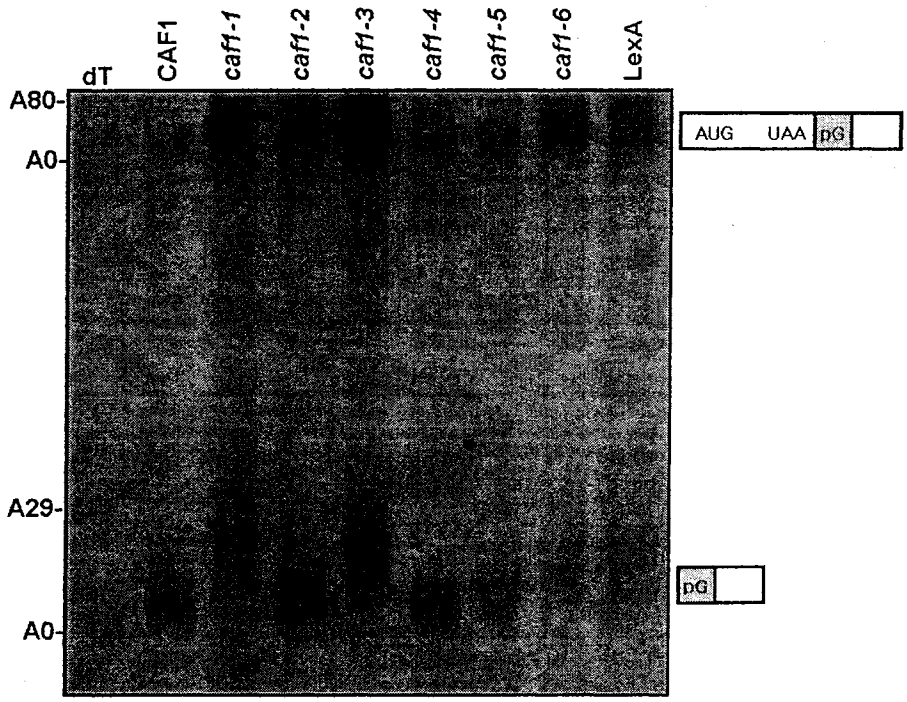


Figure 10. *caf1-1*, *-3*, and *-6* alleles are defective in deadenylation endpoint of *MFA2pG*. Northern analysis of steady state *MFA2pG* mRNA was conducted with a *caf1* background (EGY188-c1-1) in which LexA-CAF1 fusion proteins were expressed as indicated. dT refers to removal of oligo(A) by incubation with oligo(dT) followed by RNase H digestion. The approximate lengths of the poly(A) tail and migration positions of the *MFA2pG* and *pG* fragments are indicated to the left and right, respectively.

overall results indicate that the severe growth phenotypes of *caf1-1*, *-3*, and *-6* probably result from the inability of the CCR4-NOT complex to properly deadenylate in vivo.

CAF1 affects translation to a greater extent than does CCR4

The *caf1* deletion has previously been shown to be lethal with a deletion in the decapping/translational regulator DHH1 whereas the *ccr4* deletion is not lethal with *dhh1* (Maillet and Collart, 2002). In order to determine which *CAF1* allele can confer lethality with *dhh1*, we constructed a strain carrying both a *dhh1* and *caf1* deletion that was covered by a plasmid expressing a *GAL1-CAF1* gene. This strain was unable to grow on glucose growth conditions when *GAL1-CAF1* expression was shut off (not shown). Following transformation of LexA-*CAF1* plasmids encoding each of the above mutagenized *CAF1* alleles, the LexA-*caf1-1*, *-3*, and *-6* did not allow growth on glucose growth conditions whereas *caf1-2*, *-4*, and *-5* alleles allowed growth on glucose (data not shown). These data suggest the *caf1-1*, *-3*, and *-6* alleles are also defective in a non-deadenylation function. As DHH1 is known to be a decapping regulator (Coller et al., 2001) and to repress translation (Coller and Parker, 2005), CAF1 could be involved in either or both of these functions.

We first tested if CAF1 defects specifically affected the rate of protein synthesis under normal non-stress conditions. As indicated in Table 5, a *caf1* deletion reduced the rate of translation to 44% whereas an isogenic *ccr4* strain had much less effect on translation. These data suggest that CAF1 plays a particular role in translation separate from that of CCR4. We next tested if a *caf1* allele specifically affected the decapping process as compared to *ccr4*. We analyzed the *MFA2pG* RNA in this case, as the relative abundance of the *pG* fragment to that of full-length *MFA2pG* is an indicator of the

efficiency of the decapping and 5'-3' degradation process (Coller et al., 2001; Olivas and Parker, 2000). No significant difference in *pG* formation relative to that of full length *MFA2pG* was observed between *caf1* and *ccr4*, although both reduced the abundance of the *pG* fragment as compared to the wild-type strain (data not shown). These results agree with previous observations concerning *caf1* and *ccr4* effects on *pG* fragment formation (Tucker et al., 2002 & 2001) and indicate that the reduction in decapping rates observed in *ccr4 caf1* strains is apparently the result of slowed deadenylation in both cases.

Identification of DHH1 and STM1 as high-copy suppressors of a *pab1-ΔRRM2 caf1* synthetic lethality

In an analysis of poly(A) binding protein (PAB1) genetic interactions with CCR4 and CAF1, we observed that deleting the RRM2 or RRM1 domain of PAB1 was lethal with a *caf1* deletion but not with *ccr4* (summarized in Table 5). The RRM2 domain of PAB1 is important both for contacting the poly(A) tail and the translation initiation factor eIF4G (Deardorff and Sachs, 1997; Kessler and Sachs, 1998; Otero et al., 1999). The RRM1 and RRM2 deletions of PAB1 also reduced the in vivo rate of protein synthesis by 29 and 15%, respectively, whereas other RRM deletions and of the C-terminal region of PAB1 had less than 4% effects on the rate of protein translation (data not shown) and were not lethal with *caf1*. Subsequent genetic analysis showed that the *caf1-1*, *-3*, and *-6* alleles but not those of *caf1-2* or *caf1-5* were similarly lethal with *PAB1-ΔRRM2* (data not shown). These genetic interactions support the above observations that CAF1 is involved in a translation process.

Since overexpression of CCR4 is known to partially reduce the deadenylation

Table 5. Summary of genetic interactions of *caf1* and *ccr4* with translation factors.

	Rate of protein synthesis		Lethality with		
	CDC33	*cdc33-1*	*dhh1*	*PAB1-ΔRRM1*	*PAB1-ΔRRM2*
wt	100%	36%	no	no	no
caf1	44%	13%	yes	yes	yes
ccr4	67%	24%	no	no	no

Rates of protein synthesis represent the average of three to four determinants. SEM values were less than 3%. All strains were isogenic to AS319. *PAB1-ΔRRM1* and *PAB1-ΔRRM2* refer to AS319/YC505 and AS319/YC506, respectively.

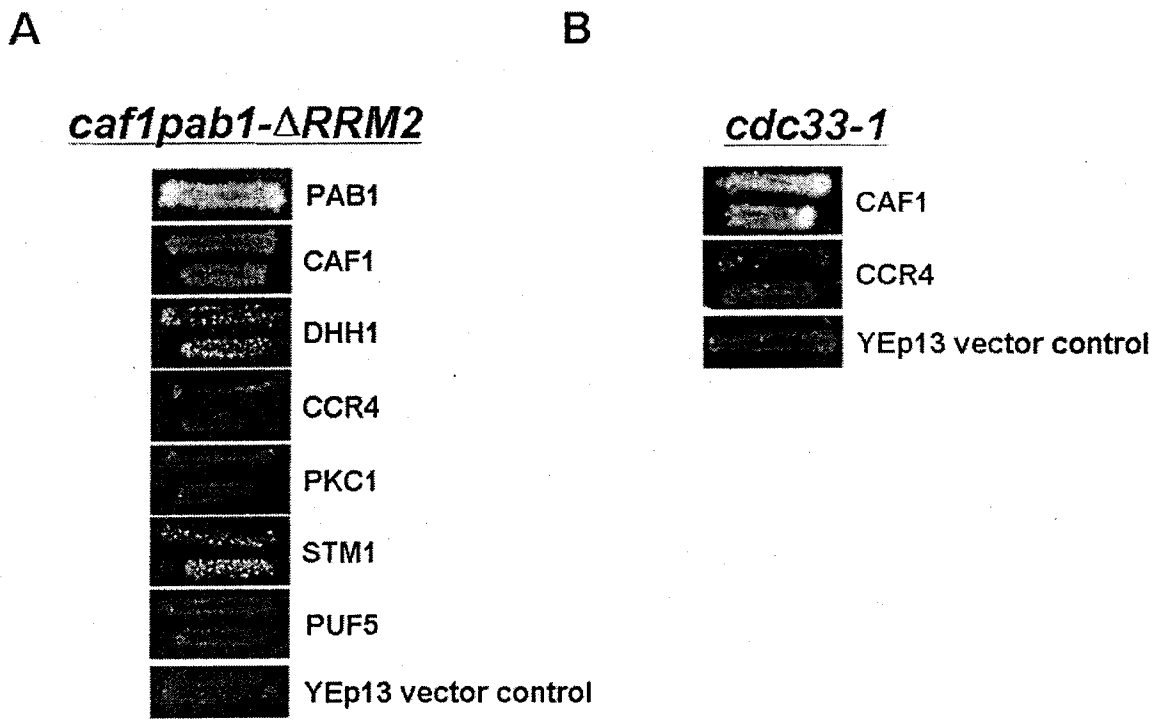


Figure 11. ***YEp13 LEU2*** **high-copy yeast library screening.** (A) Overexpression of *DHH1* and *STM1* suppress the synthetic lethality of the *caf1 PAB1-ΔRRM2* double mutant. Strains ASY319-c1-lN/YC506 (*caf1 pab1*/pYC360 (*PAB1 URA3*) pYC506 (*PAB1-ΔRRM2 TRP1*)) was transformed with the YEp13-LEU2 plasmids carrying *MPT0* (*CAF1*), *MPT1* (*DHH1*), *MPT2* (*CCR4*), *MPT3* (*PKC1*), *MPT4* (*STM1*), *MPT5* (*PUF5*) (18) and *PAB1*. Transformants were selected on minimal media lacking uracil, tryptophan, and leucine and replica plated on FOA media to lose the pYC360 plasmid. The plate was incubated at 30°C for 7 days before scanning. (B) Overexpression of *CAF1* can suppress caffeine sensitivity associated with *cdc33-1*. Strain 1881/YC360 (*PAB1 URA3*) was transformed with *MPT0*, *MPT2*, or *YEp13*.

defect in a *caf1* background (Tucker et al., 2002), one simple interpretation of the *caf1 PAB1-ΔRRM2* lethality was that a lack of deadenylation was lethal with *PAB1-ΔRRM2*. However, as *ccr4* was not lethal with *PAB1-ΔRRM2*, this simple model is probably not correct. To clarify the genetic interaction between *caf1* and *PAB1-ΔRRM2*, we conducted a genetic screen to identify genes whose overexpression could rescue this synthetic lethality. A high-copy YEp13-*LEU2* yeast genomic library was subsequently transformed into a strain carrying the *caf1 PAB1-ΔRRM2* allele. Eleven plasmids that suppressed the *caf1 PAB1-ΔRRM2* lethality were identified. Five of these plasmids contained *CAF1* and one plasmid carried *PAB1*. All of these plasmids suppressed the lethality strongly (Figure 11). Of the remaining five plasmids, two displayed moderate suppression, and of these one was found to carry the *DHH1* gene and the other the *STM1* gene. The remaining three weakly suppressing plasmids were containing OKP1, RNR1, and POL4.

Because previous analysis has identified several multi-copy suppressors of a *caf1* deletion, including that of *DHH1*, *CCR4*, *PKC1*, *STM1*, and *PUF5* (Hata et al., 1998), we tested whether overexpression of each of these different genes in a strain carrying *caf1 PAB1-ΔRRM2* and *PAB1-URA3* could suppress the *caf1 PAB1-ΔRRM2* lethality and allow loss of *PAB1-URA3* and subsequent growth on 5'-FOA media. As shown in Figure 11A, we failed to observe any high-copy suppression of *caf1 PAB1-ΔRRM2* by *CCR4*, *PKC1*, or *PUF5*. This observation provides additional genetic evidence supporting the functional participation of CAF1 in a process that is separate from the role involving CCR4.

One model to explain the role of DHH1 in suppressing the *caf1 PAB1-ΔRRM2* lethality would be that overexpression of DHH1 could compensate for the deadenylation defect caused by *caf1* mutants. As shown in Figure 12 in analyzing the steady state

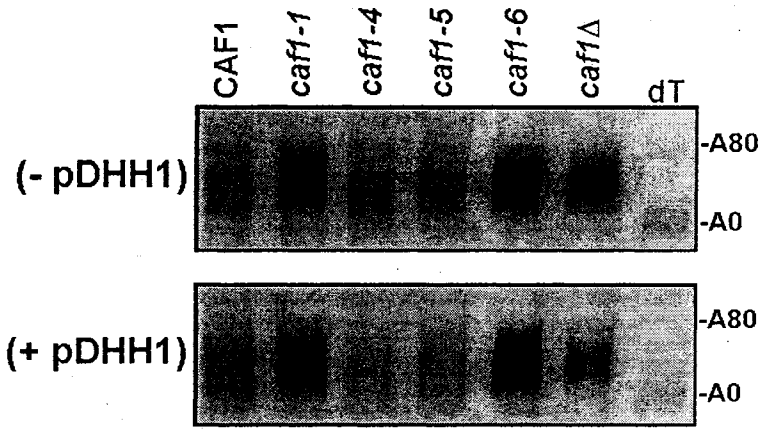


Figure 12. Overexpression of *DHH1* cannot suppress the deadenylation defects of *caf1* mutant alleles. Northern blot analyses of steady state *MFA2pG* mRNA were conducted for analyzing *MFA2pG* poly(A) tail distribution in each of the *caf1* mutant alleles with or without overexpression of *DHH1*. Flag-DHH1 was overexpressed on plasmid pRP1053. Flag-DHH1 or vector control were transformed into EGY188-c1-1 containing the different LexA- caf1 mutants and pRP485 (*MFA2pG*). Steady state mRNA samples were subjected to electrophoresis on 6%/7.5M urea polyacrylamide gels.

MFA2pG levels, overexpression of *DHH1*, however, had no effect on the deadenylation defects observed with the *caf1-1* and *-6* alleles. In both *caf1-1* and *caf1-6* at steady state *MFA2pG* mRNA does not deadenylate completely and has longer poly(A) tail lengths than the wild-type regardless of whether DHH1 was overexpressed or not. These data confirm that DHH1 functionally interacts with CAF1 through a mechanism that does not directly pertain to deadenylation. As DHH1 is involved in translation processes (Coller et al., 2001; Coller and Parker, 2005) and STM1 can interact with the ribosome and eIF4E (Gavin et al., 2002), we determined whether overexpression of CAF1 or CCR4 could complement a defect in translation factor eIF4E (*cdc33-1* allele). As shown in Figure 11B, overexpression of CAF1 but not that of CCR4 could complement the caffeine sensitivity displayed in a strain carrying the *cdc33-1* allele. As *cdc33-1* is specifically defective in binding the 5' mRNA cap (Altman and Trachsel, 1989), these results provide further support for a functional role for CAF1 in translation distinct from its role in deadenylation.

A translation defect in PAB1 specifically reduces deadenylation when coupled with a *caf1* deletion.

The above results implicate CAF1 in affecting translational process. As the primary lesion of a *CAF1* deletion is decreased deadenylation, it might be that CAF1 serves to communicate a positive effect between deadenylation and translation. However, previously it has been shown that defects in translation initiation factors accelerated deadenylation (Schwartz and Parker, 1999), implying that they play a negative role in deadenylation. In order to determine if other aspects of translation played required roles for deadenylation, we examined the effects of two defined PAB1 defects in translation on

deadenylation: PAB1-134 and PAB1-184. PAB1-134 has been shown to reduce 5'cap-dependent translation in vitro whereas PAB1-184 affected poly(A)-dependent translation, apparently through reduced binding to eIF4G (Otero and Sach, 1999).

Using the analysis of the steady state levels of *GAL1* mRNA as an initial determination of the effects of these PAB1 variants on deadenylation, we found that neither PAB1-134 nor PAB1-184 affected the distribution of steady state poly(A) tail lengths as compared to wild-type PAB1 (Figure 13A). In contrast, *ccr4* or *caf1* deletions displayed much longer poly(A) tail lengths (Figure 13A), indicative of slowed deadenylation (Tucker et al., 2001). Subsequent analysis of PAB1-134 and PAB1-184 effects on the deadenylation rate of the *MFA2pG* mRNA using pulse chase analysis indicated that neither affected the deadenylation process (data not shown). However, when PAB1-134 was combined with the *caf1* deletion, the steady state analysis of *GAL1*-L or *GAL1*-S showed that the poly(A) tail lengths were much longer that with *caf1* alone, as if there were a complete block in deadenylation (Figure 13A; data not shown). In contrast, PAB1-134 had no effect on the poly(A) tail length when combined with a *ccr4* deletion (Figure 13A). PAB1-184, as comparison, did not show this effect with *caf1* (Figure 12A). Analysis of the actual deadenylation rate for *GAL1*-L showed that a *caf1 PAB1-134* background caused a severe slowing of the rate of deadenylation (0.7 A's/min compared to 3.7 A's/min for wild type *PAB1* and 2.2 A's/min for *caf1*) (Figure 13B). These observations indicate that some defects in translation can cause corresponding defects in deadenylation and that proper translation coupled with CAF1 is required for complete deadenylation by CCR4 (see Discussion).

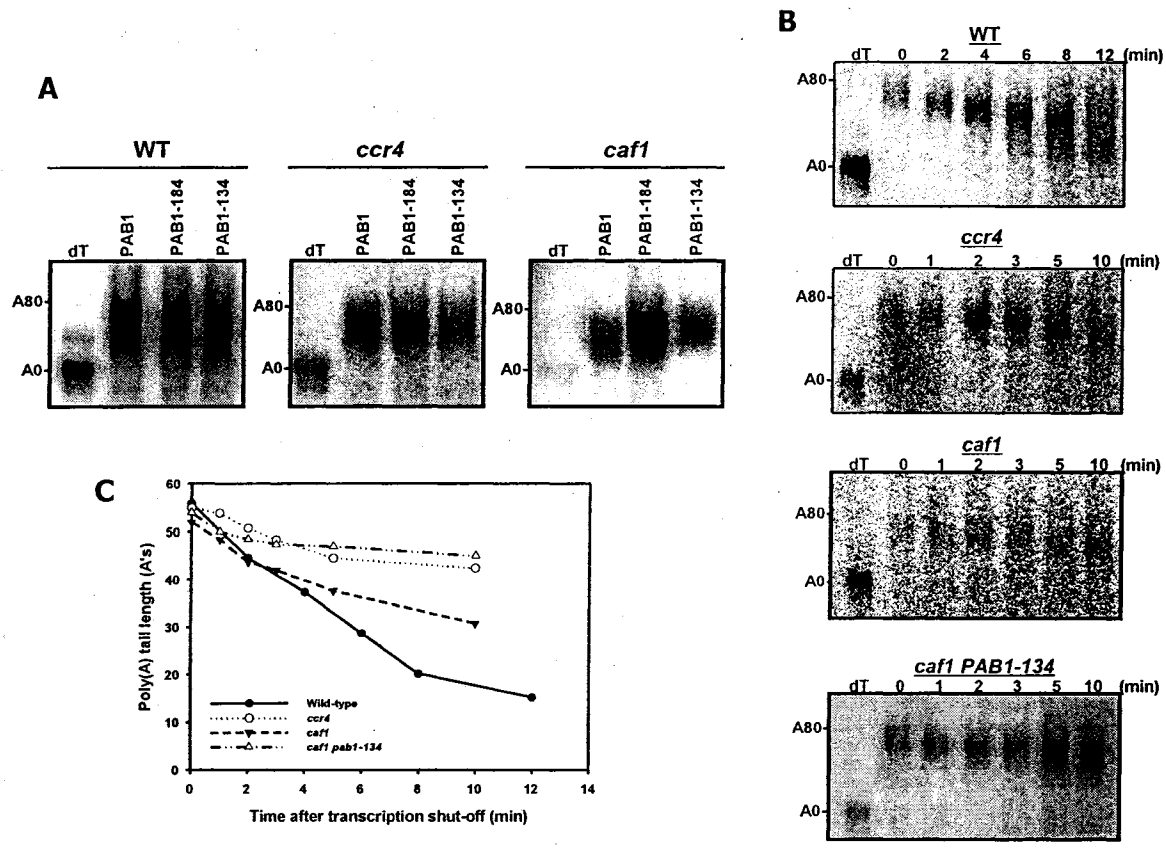


Figure 13. PAB1-134 blocks deadenylation in a *caf1* background. A. Steady state mRNA analysis of *GAL1* was conducted as described in Figure 6. wt-AS319; *ccr4*-AS319-1a-uN; *caf1*-AS319-c1-lN. B. Pulse-chase analysis of *GAL1*-L mRNA deadenylation was conducted as described in Figure 4. PAB1-134 refers to AS319/YC545 and PAB1-184 refers to AS319/YC538. YC545 expresses the PAB1 protein with residues 134HPD137 replaced with DKS and YC538 has residues 184DAL187 of PAB1 replaced with EKM (29).

Discussion

It has been shown that the CAF1 C-terminal domain purified from *E. coli* exhibits an exonuclease activity with a poly(A) preference (Daugeron et al., 2001; Thore et al., 2003). Our previous mutation studies of CAF1 established, however, that its RNase function is not required for the in vivo deadenylation process (Viswanathan et al., 2004). This discrepancy prompted us to identify the functional domains that are critical for CAF1 to perform its role in mRNA turnover. By targeting only those sequences absolutely conserved amongst all CAF1 orthologs but not present in other members of the DEDDh family of nucleases, we sought to identify CAF1-specific functions. This methodology led us to define several separable functional regions in the CAF1 protein.

HA immunoprecipitation (data not shown) and FLAG pull-down analyses with *CAF1* mutants used in this study showed that three amino acids (213FRS215) deleted in *caf1-2* are required for the CAF1 interaction with CCR4. As mammalian CAF1 can also bind yeast CCR4, it would be expected that the CAF1 residues involved in this contact have been retained evolutionarily. Residues 213-215 are found in a loop structure whose structure was not apparently fixed enough to be determined in the X-ray analysis of CAF1 (Thore et al., 2003). The caf1-2 protein displayed only a weak caffeine sensitive phenotype and intermediate effects on deadenylation in vivo as compared to a *caf1* deletion or to mutations in other regions of the CAF1 protein that were still capable of binding CCR4. It appears, therefore, that CAF1 binding to CCR4 is not the central function of CAF1 for deadenylation. This conclusion is in agreement with results from our previous analysis wherein mutations in CCR4 that significantly reduced CCR4 binding to CAF1 appeared phenotypically wild-type (Clark et al., 2004).

In contrast to *caf1-2*, the most important regions for CAF1 function *in vivo* were identified by the *caf1-1*, *-3*, and *-6* alleles. These three *caf1* mutant alleles displayed severe defects in poly(A) shortening like that of a *caf1* deletion and were as caffeine sensitive as the *caf1* deletion. Three observations suggest that *caf1-1* and *caf1-3* alleles are equivalent physically and functionally. First, the X-ray crystal structure showed that *caf1-1* and *caf1-3* alleles are located in the same region where two β sheets (β1 and β4) are aligned side by side (Figure 2A). Second, yeast cells carrying either of the two alleles displayed a dominant negative growth phenotype. Third, both alleles showed similar *GAL1* deadenylation rates and defects in *MFA2pG* endpoints. These observations indicate that the *caf1-1* and *caf1-3* alleles define a novel functional region of yeast CAF1 required for its cellular function. The *caf1-6* allele, deleting residues 340DFE342, define a third functional region of CAF1. Phenotypes associated with *caf1-6* are nearly identical to those observed for *caf1-1* and *-3*, although the location of the *caf1-6* mutation is on the opposite site of the CAF1 protein as that of *caf1-1* and *-3*. Whether the putative contacts made by the caf1-6 region (α8 helix) are to the same factors as presumed to contact the β1 and β4 sheets remain to be determined. At least, both regions and these putative contacts are central to CAF1 *in vivo* function.

In that we also observed that none of our *CAF1* alleles severely affected *in vitro* CCR4 deadenylation activity regardless of whether the alleles blocked CCR4 binding to CAF1 (*caf1-2*) or not, we conclude that CAF1 does not directly regulate CCR4 activity in vitro. This is in agreement with our previous observations (Chen et al., 2002; Clark et al., 2004). All of the *CAF1* alleles interacted equivalently well with NOT1, further suggesting that the identified roles for CAF1 in vivo are not defined through this interaction either.

What, then, is the role for CAF1 *in vivo*? Several previous observations and those made herein suggest that CAF1 affects non-deadenylation processes in addition to its effects on deadenylation. These non-deadenylation effects could be on decapping or translation. However, we and others (Tucker et al., 2002 & 2003) have observed that a *caf1* deletion does not specifically affect decapping *in vivo* as compared to the effects displayed by *ccr4*. A role, instead, for CAF1 in translation separate from CCR4 is supported by a number of observations. First, a *caf1* deletion displays synthetic lethalities with defects in translational regulators that *ccr4* deletion does not. A *caf1* deletion, but not that of *ccr4* is lethal with *dhh1*, a decapping regulator that is required for translational repression (Maillet and Collart, 2002; Coller and Parker, 2005; data not shown). The *caf1-1*, *-3*, and *-6* alleles also displayed this lethality with *dhh1*. In addition, *caf1* and the *caf1-1*, *-3*, and *-6* alleles were also lethal with *PAB1-ΔRRM1* and *PAB1-ΔRRM2*, a phenotype not observed with *ccr4*. Only these two deletions in PAB1 affected translation. Second, we found that *caf1 PAB1-ΔRRM2* lethality can be suppressed by overexpression of two factors linked to translation, DHH1 and STM1. As overexpression of CCR4 cannot suppress this lethality, although it can suppress *caf1* deadenylation defects (Tucker et al., 2002), it is clear that deadenylation defects alone are not contributing to *caf1 PAB1-ΔRRM2* lethality. In vivo immunological and biochemical studies with STM1 have shown that it is localized in the cytoplasm and associated with primarily 80S monosomes and polysomes (Van Dyke et al., 2004). Two-hybrid data further suggest physical interaction of STM1 with the translation initiation factor eIF4E (Gavin et al., 2002). Third, we showed that a *caf1* deletion exerts more severe effects on translation that does *ccr4*. Fourth, we showed that overexpression of CAF1 but not CCR4 can suppress the caffeine sensitivity associated with a cap binding defect in eIF4E.

As CAF1 is required for the deadenylation process, the above observations supporting a role for CAF1 in translation separate from its contact to CCR4, suggest that there may be a required interplay between translation and deadenylation. Previously, defects in translation initiation factors were found to enhance deadenylation, not slow it (Schwartz and Parker, 1999). We have found, however, that the defect in PAB1-134 that reduces cap-dependent translation *in vitro*, is required for deadenylation in a *caf1* background. This result implies that CAF1 and the translation function defined by PAB1-134 are both required for the full CCR4 deadenylation process. PAB1-134 did not affect deadenylation in a *ccr4* background suggesting that its effects are not through PAN2. Therefore, instead of translation strictly inhibiting deadenylation, these results suggest an additional positive role for translation in deadenylation. CAF1 may be the mediator of this role, as it has a number of genetic and functional interactions with translation factors and is required for deadenylation. CAF1 may, for instance, monitor the translation status and, in turn, communicate that to the CCR4-NOT complex. The exact role for CAF1 in these two processes may require the development of in vitro systems that are both translationally competent and whose mRNA deadenylation is CCR4-NOT dependent.

CHAPTER II

EFFECT OF PUF3 AND TRANSLATION INITIALTION FACTORS ON mRNA DEADENYLATION

Introduction

Post-transcriptional control in eukaryotic cells is essential for proper regulation of gene expression. The post-transcriptional processes that regulate the turnover of an mRNA and the control of its translation in the cytoplasm are the major sites at which such coordinated gene expression control occurs. Such control is commonly mediated by mRNA binding proteins, many of which are specifically targeted to a specific gene sequence in 3' untranslated region (3'-UTR) (Derrigo et al., 2000; Grzybowska et al., 2001; Mazumder et al., 2003).

The first two members of the RNA-binding protein family were *Drosophila melanogaster* Pumilio and *Caenorhabditis elegans* FBF; hence, the group is now referred to as PUF (Zamore et al., 1997) or Pum-HD (Zhang et al., 1997) proteins. Pumilio from *Drosophila melanogaster* promotes anterior/posterior patterning of the early embryo by binding the 3'-UTR of hunchback mRNA and subsequently repressing its translation (Murata and Wharton, 1995) and stimulating its deadenylation (Wreden et al., 1997). *C. elegans* FBF regulates the germline swich from spermatogenesis to oogenesis by repressing *fem-3* (Zhang et al., 1997). PUF proteins in yeast regulate aging, mitochondrial function and mating-type switching (Kennedy et al., 1997; Olivas and

Parker, 2000; Tadauchi et al., 2001) whereas *Dictyostelium* PufA promotes vegetative growth (Souza et al., 1999).

The yeast *Saccharomyces cerevisiae* contains six members of PUF protein family (PUF1 through 6), which are containing 6-8 pumilio repeats (Zamore et al., 1997). Yeast and higher eukaryotic PUFs have been shown to bind 3'-UTR sequences and to either stimulate the degradation of target mRNA or repress translation process (Olivas and Parker, 2000; Jackson et al., 2004; Chagnovich and Lehnmann, 2001; Wreden et al., 1997; Wharton et al., 1998; Gu et al., 2004). To date, only three of the yeast PUFs have identified the roles in controlling specific RNA targets. PUF3 binds the 3'-UTR of *COX17* mRNA and promotes its deadenylation and subsequent decay (Olivas and Parker, 2000), PUF5 binds to the 3'-UTR of the *HO* mRNA, repressing its expression and stimulating its decay (Tadauchi et al, 2001), and PUF6 binds the 3'-UTR of the *ASH1* mRNA to regulate its translation and localization (Gu et al., 2004). In addition to these target mRNAs, microarray analysis utilizing affinity tagging of five PUF family of protein has identified several hundred candidate mRNA targets and the mRNAs targeted by each PUF. These mRNA encode functionally related families of proteins indicating that combinatorial binding of mRNAs by specific RNA binding proteins is a critical tool for efficient reprogramming of gene expression (Gerber et al., 2004; Keene, 2003; Murata et al., 1995). For example, PUF3 was specifically bound to mRNAs encoding mitochondrial proteins, PUF1 and PUF2 were associated with mRNA encoding membrane-associated proteins and PUF4 and PUF5 were selectively interacted with mRNAs encoding nuclear components (Gerber et al., 2004).

PUF proteins contain 6-8 pumilio repeats (Zamore et al., 1997) through which 3'-UTR of target mRNA binds. The binding sequences of all RNA targets analyzed to

date consists of at least a UGUR sequence and 4-6 additional flanking sequences (Wickens et al., 2002; White et al., 2001; Zamore et al., 1997). The formation of PUF protein complexes on 3'-UTRs also requires additional sequences, due to other RNA-binding proteins that are working partners of PUF proteins (Sonoda et al., 1999).

Materials and Methods

Yeast strains and growth conditions

The genotypes of the *S. cerevisiae* strains used are listed in Table 5. Yeast strains were grown on synthetic medium supplemented with 2% raffinose. In order to conduct transcriptional pulse-chase analysis, *GAL1* promoter was induced by 2% galatose and repressed by 4% glucose.

In vivo transcriptional pulse-chase analyses

Transcriptional pulse-chase experiments of *COX17* RNA were performed essentially as described (Decker and Parker, 1993; Muhlrad and Parker, 1992) on strains RP840 (wild-type), RP840-1a (*ccr4*Δ), RP1241 (*puf3*Δ), RP1241-1a (*ccr4 puf3*) and PP2 (*puf3 pan2*). These strains contain the plasmid pRP1007, in which the *COX17* gene is under the control the *GAL1* promoter. Transcription was induced for 10~20 min by the addition of galatose and then rapidly repressed by adding glucose. Poly (A) tail lengths were monitored by the cleavage of *COX17* mRNA just upstream of the stop codon using RNAseH reactions with the probe *COX17*/dn (5'-GCCATAACCCTTCATGCACTC-3') as described (Olivas and Parker, 2000). Digested and purified total RNA was separated

on 6% denaturing polyacrylamide gels at 250V for 4.5 hours and transferred to nylon membrane for probing with radiolabeled *COX17*/3'-end (5'-GGTTGTCGGCAGACTGTCAC-3') (Olivas and Parker, 2000).

For the snapshot analysis of *COX17* mRNA, yeast strains were grown to mid-log phase (OD_{600} of 0.7), transferred to fresh media. Cells were harvested at 20 minutes after induction and 5 minutes after repression.

Transcriptional pulse-chase experiments of *GAL1* gene were conducted as described in Chapter 1 except for the growth condition. Briefly, the temperature sensitive cells were grown to mid-log phase in medium containing 2% raffinose at permissive temperature (25°C), harvested, and resuspended in a fresh media prewarmed to a non-permissive temperature (38°C). After 20 min in a shaking incubator, galactose (2% final conc.) was added to induce transcription and finally glucose (4% final conc.) was added to shut off transcription.

Table 6. Yeast strains

Strain	Genotype
RP840	*MAT*a *his4-539 leu2-3 112 trp1-1 ura3-52 cup1::LEU2/PGK1pG/MFA2pG*
RP840-1a	Isogenic to RP840 except *ccr4::URA3*
RP1320	*MAT*a *his3Δ1 leu2-3, 112 lys2-201 trp1 ura3-52 cup1::LEU2/PGK1pG/MFA2pG tif4631::LEU2*
RP1320-1a	Isogenic to RP1320 except *ccr4::URA3*
RP1326	*MATα leu2 prt1-63 trp1 ura3 cup1::LEU2/PGK1pG/MFA2pG*
RP1326-1a	Isogenic to RP1326 except *ccr4::URA3*
RP1241	*MAT*a *his4-539 leu2-3 112 trp1-1 ura3-52 cup1::LEU2/PGK1pG/MFA2pG puf3::Neo*
RP1241-1a	Isogenic to RP1241 except *ccr4::URA3*
PP2	

Results and Discussion

PUF3 affects both CCR4 and PAN2 in *COX17* mRNA deadenylation

It has been reported that PUF3 specifically promotes degradation of *COX17* transcript by enhancing deadenylation and subsequent turnover through binding the 3' UTR of *COX17* mRNA (Olivas and Parker, 2000). Since the CCR4-NOT complex was revealed to be the major deadenylase complex in yeast, our lab tested if physical contact exists between PUF3 and CCR4-NOTs. Using GST-PUF3 and FLAG-PUF3, we have established that PUF3 protein physically contact CCR4-NOT complex in vitro and in vivo (Yeuh-chin Chiang, personal communication) suggesting that PUF3 acts through the CCR4-NOT complex to accelerate degradation.

To test the effect of PUF3 on CCR4 activity in vivo, we constructed *ccr4* and *pan2* deletion strains in combination with *puf3*Δ. In this way, we would be able to determine if PUF3 affected CCR4 and/or PAN2 deadenylation of *COX17*. We first analyzed the poly(A) distributions and deadenylation status of *COX17* in a short time period by performing Northern blot analysis. In this experiment, yeast cells were harvested after 20 minute activation and 5 minute repression of COX17 mRNA transcription (See Materials and Methods). Total RNA from those strains were isolated and subjected in a Northern blot analysis after RNase H digestion (See Materials and Methods). As described previously (Olivas and Parker, 2000), *COX17* mRNA poly(A) tail distribution in wild-type is weighted toward longer tails of 40~60 A residues as compared to 20~45 A residues in *puf3* deletion strains (Figure 14). This observation indicates that PUF3 is necessary to modulate the poly(A) tail status of *COX17* mRNA.

For the degradation pattern of *COX17* mRNA within 5 minutes, all mutant strains showed slower degradation except wild-type. Notably, *puf3 ccr4* double mutants seemed to have severe defect in deadenylation process.

To determine the deadenylation rates of *COX17* mRNA in these mutant strains, we conducted transcriptional pulse-chase experiments. In this experiment, *COX17* mRNA is transcribed from a plasmid under the control of *GAL1* promoter. As described previously (Jackson et al., 2004), the poly(A) tails of *COX17* mRNA from wild-type strain were deadenylated completely within 2-4 minutes, and all transcripts disappeared within 10 minutes. In contrast, deadenylation rates of *COX17* in *puf3*Δ strain, as previously demonstrated (Olivas and Parker, 2000) slowed considerably as compared to that of wild-type. The average rates of deadenylation of *COX17* in wild-type and *puf3*Δ were 5.0 and 1.3 A's/minute respectively. The *ccr4* deletion stain also displayed a slower rate of deadenylation such that *COX17* transcripts did not become fully deadenylated until 20 min. Both of the deadenylation rates of *ccr4*Δ and *puf3*Δ strains were 1.3~1.4 A's/min suggesting that these proteins are equally important for the *COX17* deadenylation process. We next tested if PUF3 protein also affects *COX17* deadenylation by PAN2, a deadenylase involving in the initial poly(A) tail trimming, by using *ccr4*Δ *puf3*Δ double deletion stain. As we predicted in snapshot analysis (Figure 15), deadenylation of *COX17* mRNA was completely blocked suggesting that PUF3 also affects PAN2 for *COX17* deadenylation. On the other hand, the *puf3 pan2* double deletion strain displayed slower deadenylation as compared to the *puf3*Δ strains. It is interesting to note that *puf3 pan2* strain may lost poly(A) tail length control because even

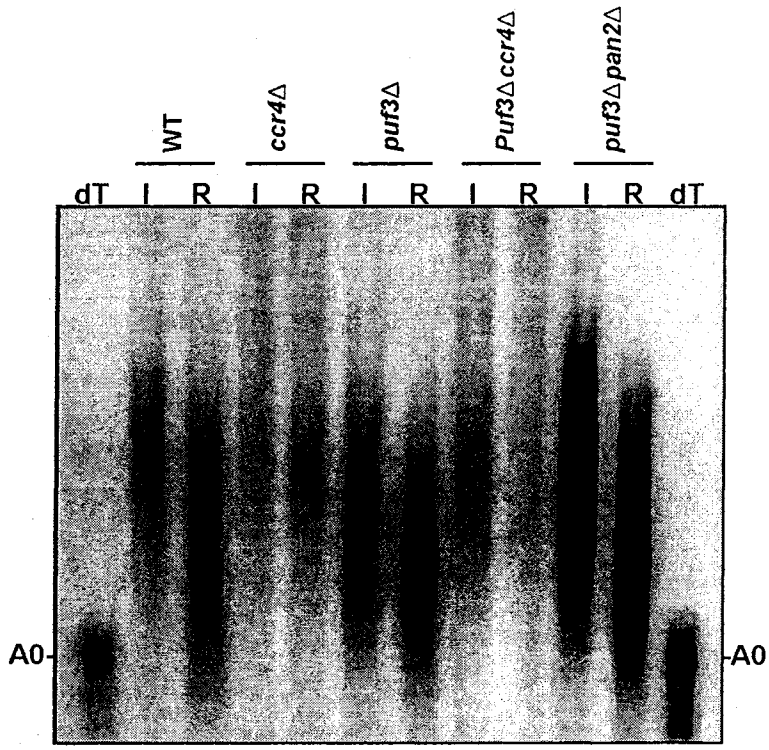


Figure 14. Snapshot of poly(A) tail distribution of *COX17* mRNA. Transcription of *COX17* mRNA was induced for 20 min by adding galactose in growing media (I) and repressed for 5 min by the addition of glucose (R). Total RNA from wild-type (WT) or indicated deletion strains was cleaved utilizing RNase H reactions to produce 3'-UTR-poly(A) tail fragments and analyzed in Northern blots (see Materials and Methods). dT refers to deadenylated *COX17* RNA by oligo dT and subsequent RNase H digestion.

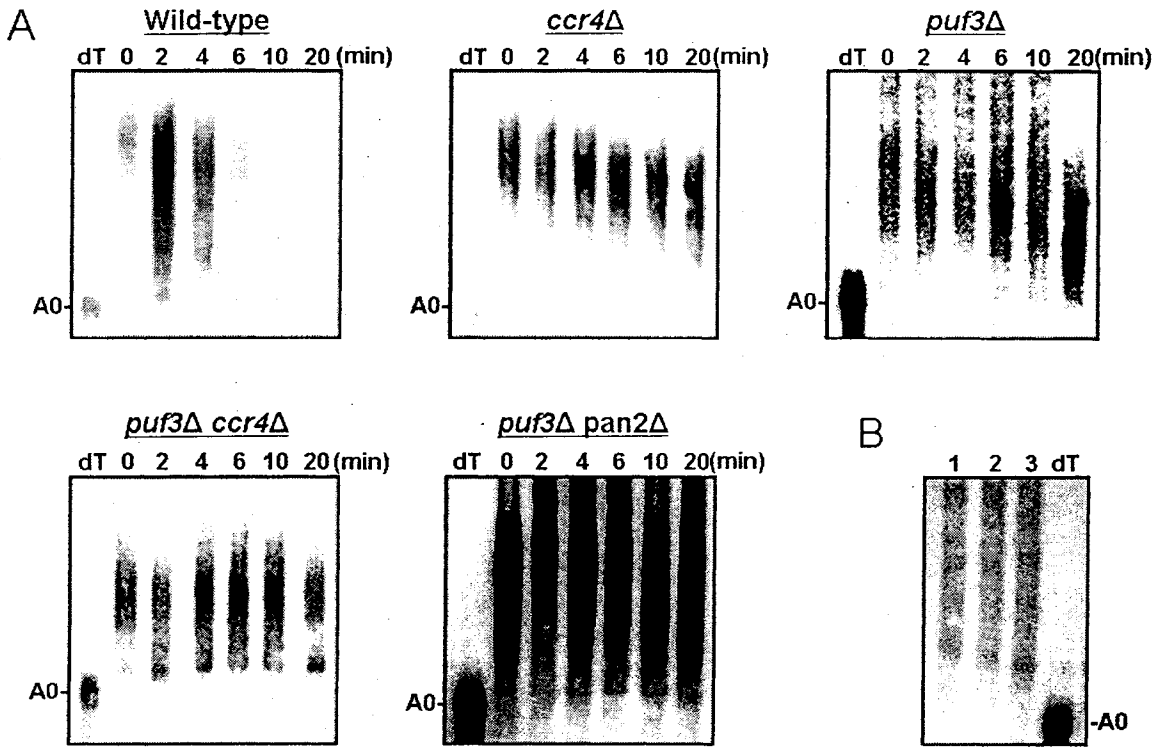


Figure 15. PUF3 affects *COX17* deadenylation both by CCR4 and PAN2. (A) Shown are the Northern blots of *COX17* transcriptional pulse-chase analyses from wild-type and indicated mutant strains; *ccr4*Δ, *puf3*Δ, *ccr4*Δ *puf3*Δ, and *puf3*Δ *pan2*Δ. The numbers on the blots are minutes following transcriptional repression. dT lane in each blot corresponds to RNA from the 0 min time point in which the poly(A) tail was removed by oligo dT and RNase H. (B) The numbers above the blot denotes minutes after transcriptional activation from *puf3*Δ *pan2*Δ strain background.

one minute induction of *COX17* transcription failed to get a proper pulse of *COX17* mRNA containing 45~60 poly(A) tails (Figure 15B). This phenomenon may due to the involvement of PUF3 in poly(A) tail length control by affecting PAN2 function. Further analysis will be needed to verify this hypothesis.

Defects in translation initiation factors affect PAN2 deadenylation

Translation initiation defects such as in *prt1-63* or deletion of eIF4G accelerate deadenylation (Schwartz and Parker, 1999), but it has not been known if those defects affect both CCR4 and PAN2. If translation initiation factors (TIFs) are affecting some general feature of mRNP structure, then defects in TIFs would also be expected to affect PAN2 function. To test this hypothesis, we used yeast strains harboring defect in TIFs such as eIF3 (*PRT1*) and eIF4G1 (*TIF4631*) in combination with *ccr4* deletion. We first tested if defects in *PRT1* (*prt1-63*) and *TIF4631* (eIF4GΔ) accelerated deadenylation as described previously (Schwartz and Parker, 1999). As shown in Figure 16, for the wild-type strain it took about 5 min for *GAL1*-L poly(A) tails to be shortened to the oligo(A) form. Whereas in the *tif4631* and *prt1-63* backgrounds, to oligo(A) form appears in about 2 min. Calculation of the rate of deadenylation indicates that deadenylation for *tif4631* occurred at about 9.7 A's/min and for *prt1-63* at about 12.5 A's/min which are about 1.5 and 2 fold as fast compared to wild-type (6.3 A's/min) (Figure 16; Table 7). These results agree well with previous decay analyses with *MFA2* and *PGK1* mRNAs in *tif4631* and *prt1-63* bacgrounds (Schwartz and Parker, 1999). Faster deadenylation by PAN2 was also observed in *tif4631* and *prt1-63* with a *ccr4* deletion, when only PAN2 was active, as compared to *ccr4*Δ. Changes in the deadenylation rate were increased about 1.6 to

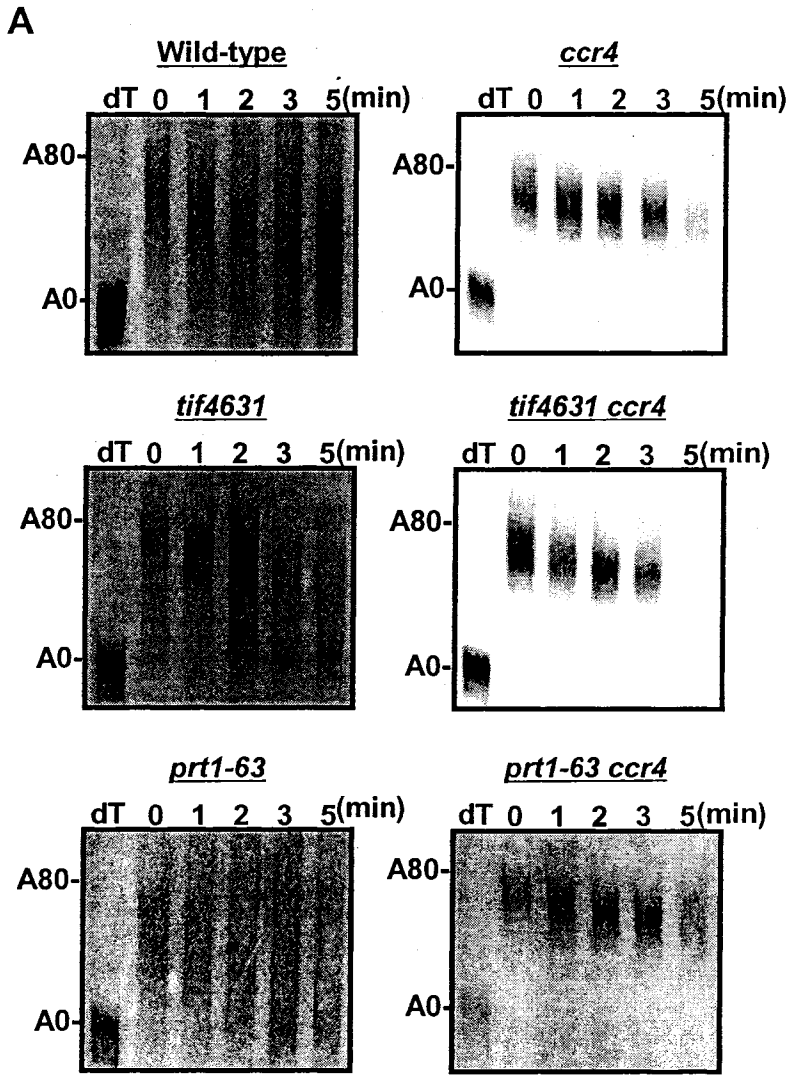
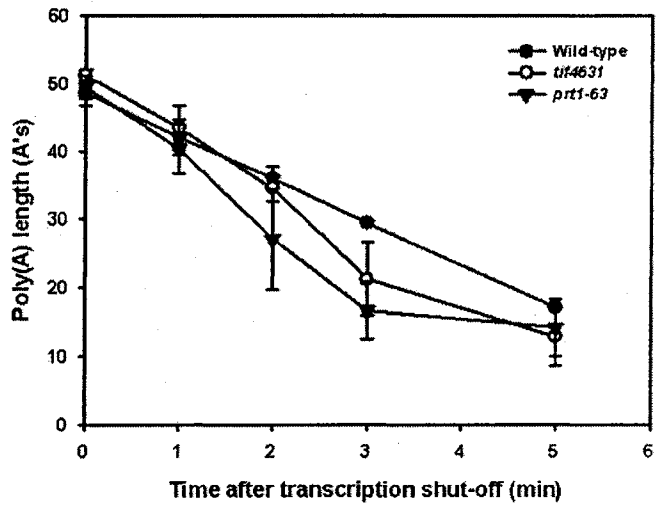


Figure 16. Lesions in translation initiation factors affect deadenylation both by CCR4 and PAN2. (A) Nothern blots of transcriptional pulse-chase experiments examining the deadenylation of *GAL1*-L mRNA in translation initiation mutants are shown. Minutes following transcription repression are given above each blot. The numbers after the A's in the cartoons represent the range of poly(A) tail sizes. dT lanes denotes *GAL1*-L mRNA whose poly(A) tail have been completely removed by cleavage with RNase H and *GAL1* probe 3 oligo nucleotide (see the Materials and Methods in Chapter I). (B) Graphical representation of *GAL1*-L deadenylation in mutant strains shown in A.

B



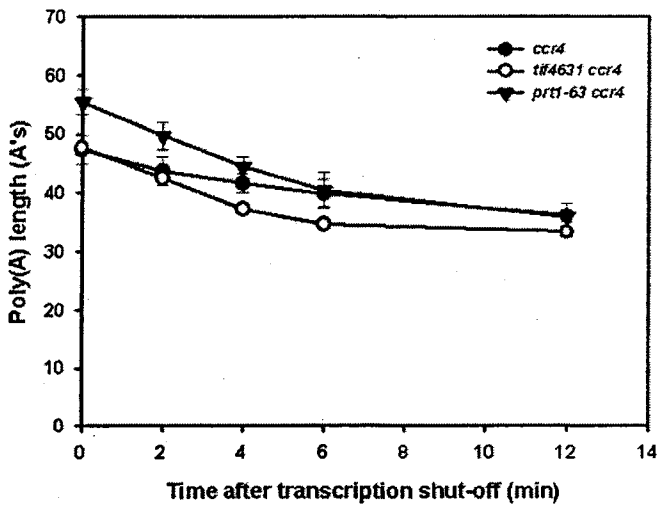


Table 7. Effects of translation initiation factors on deadenylation of *GAL1*-L mRNA

Strain	Deadenylation rate (A's/min ± SEM)
Wild-type	6.3 ± 0.4
tif4631	9.7 ± 1.3
prt1-63	12.5 ± 2.2
*ccr4*Δ	0.9 ± 0.1
*tif4631 ccr4*Δ	1.4 ± 0.3
*prt1-63 ccr4*Δ	1.6 ± 0.0

1.7-fold as compared to the ccr4 control strain (Figure 16; Table 7).

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